Vol. 3, No. 1 JULY, 1923

THE JOURNAL OF BIOCHEMISTRY

EDITED BY

SAMURO KAKIUCHI

Professor in the Tokyo Imperial University

Reprinted by
TOA BOOK EXPORTS, INC.

Tokyo Kosho Kaikan Building
Kanda Ogawa-cho 3-22, Tokyo
CABLE: Toaperiodical, Tokyo
PHONE: Tokyo 291-1448

THE JOURNAL
OF
BIOCHEMISTRY

WITH THE COOPERATION OF

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VOLUME III
TOKYO
1924

PUBLISHED BY THE JOURNAL OF BIOCHEMISTRY

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No. 1, JULY, 1923.

No. 2, JANUARY, 1924.

No. 3, APRIL, 1924.

ON THE GERMICIDAL ACTION OF HYDROSOL OF COPPER.

By

MICHIO KUSUNOKI.

(From the Biochemical Laboratory, Institute of Medical Chemistry, Tokyo Imperial University. Director: Prof. S. Kakiuchi.)

(Received for publication, March 1, 1923)

I. Introductory.

After the publication of my first paper on the germicidal action of hydrosol of silver, I have found a number of interesting reports regarding to the oligodynamic action of heavy metals. It is especially worthy of notice, that, according to the summary given by K. Süpfler (1922), Nägeli had given a postulate already in 1893 that the oligodynamic action of heavy metals, such as Cu, Ag, Hg, Zn, Fe, etc. ought to be ascribed to the metal ions produced from the metals by the presence of oxygen and carbon dioxide in the atmosphere. Although this assumption has been made probable by the work of such investigators as Rankin (1911) Spiro (1915, 1916), Acél (1920) Doerr (1920, 1921), Wernicke and Sordelli (1921), Doerr and Berger (1922), and others in the experiments on metal plates, wires and oxides, the colloidal solution of pure metals has not yet been used in this connection and as to the bearing of colloidal metallic particles in the bactericidal action there is no convincing explanation afforded.

In the previous paper (1922) the germicidal action of silver hydrosol was reported by the author, the rèsume being given as follows; the germicidal action of colloidal silver is only due to the silver ion ionised from silver oxide and carbonate produced from silver sol by the presence of ordinary

air, while the colloidal silver particle itself remains almost without any action. The author studied further the bactericidal action of copper hydrosol, the result of which will be reported in this paper.

II. The Material and Method.

Copper hydrosol. Copper hydrosol used in my experiments was the copper-Yemorisol obtained from Tomoda in Tokyo.

The copper-Yemorisol is prepared by the electrical method from metallic copper and its colour is green and clear, the content in copper is about 0.05 %.

Bacterial Suspension. The bacterial suspension was prepared by suspending a loop of coli bacilli in about 20 cc. of distilled water.

The technique. To 10–12 cc. of copper sol in each of the flasks of about 20 cc. capacity one drop of bacterial suspension was added, shaken well and allowed to stand for a given time. After placing 0.5–1 cc. of the mixture in Petri dishes about 12 cc. of liquefied agar of about 50° C were poured into each of them. The plates, thus obtained, were incubated at 37°C for 24 hours, the number of colonies was then counted, and recorded.

At first in this experiment hydrogen sulphide water was applied before inoculation in culture medium to avoid a successive action of copper on bacteria but it was found that no great difference was observable in the results between the cases with and without H_2S. As it is not our purpose to determine the absolute value of the bactericidal activity of the material, but only desired to prove the existence of germicide in it, the addition of H_2S was omitted in the following experiments.

III. Germicidal Action of Copper-Yemorisol.

The copper-Yemorisol was diluted with distilled water in various grades and each bactericidal action was tested using the above mentioned technique. The results are tabulated as follows.

TABLE I.

Number of colonies after treatment of bacteria with Yemorisol.

Minutes.	Dilution of copper-Yemorisol.				
	1	3	9	27	H_2O
15′	240	680	660	800	∞
30′	160	200	270	430	∞
45′	50	100	130	220	∞
60′	10	54	65	82	∞
90′	—	11	23	54	∞

IV. Germicidal Action of Dialysate.

Every 10 cc. of copper-Yemorisol of different concentration was poured into collodion thimble placed in a Erlenmeyer flask containig 100 cc. of distilled water and dialysed for 24 hours. Four dialysates were thus obtained. All of them exerted a remarkable bactericidal power. Their action was, however, extremely reduced by the addition of the hydrosulphuretted water to the dialysates; as can be seen from the following table.

TABLE II.

Number of colonies, when bacteria were treated with the mixture of 10 cc. of dialysates and 2 cc. of distilled water.

Minutes	Dilution of copper-Yemorisol				
	1	3	9	26	Control
15′	1000	1600	∞	∞	∞
30′	200	440	∞	∞	∞
45′	120	200	1300	∞	∞
60′	28	40	900	1500	∞
90′	—	5	550	980	∞

Number of colonies, when bacteria were treated with the mixture of 10 cc. of dialysates and 2 cc. of H_2S solution.

Minutes.	Dilution of copper-Yemorisol.				
	1	3	9	27	Control
15′	∞	∞	∞	∞	∞
30′	∞	∞	∞	∞	∞
45′	∞	∞	∞	∞	∞
60′	∞	∞	∞	∞	∞
90′	∞	∞	∞	∞	∞

It is obvious from the above table that this bactericidal substance was diffusible through membrane, and that its action was remarkably reduced on addition of H_2S, this indicating clearly copper ion as the carrier of the action. As regards the mechanism of production of copper ion we were reminded from the results with silver sol that the existence of oxygen and carbon dioxide in the atmosphere must be the main cause. To ascertain this relation the following experiment was conducted.

V. The Effect of the Nature of Atmospheric Gases on Germicidal Action.

On comparing the bactericidal power of the dialysates obtained by dialysis in atmosphere of five different gases—oxygen, air free from carbon dioxide, hydrogen, ordinary air and carbon dioxide—most of them were strongly bactericidal and no recognizable difference was found among them, only with an exception of the one obtained in the medium of carbon dioxide, the action of which was far inferior to the others as shown in the next table.

TABLE III.

Duration of exposure.	O_2	Air-CO_2	H_2	ord. air	CO_2
15′	1700	1600	2000	1600	∞
30′	200	240	250	160	∞
45′	45	68	48	40	∞
60′	9	10	5	6	1200
90′	—	—	3	—	800

During the course of repeated dialysis the green colour within collodion thimbles faded off gradually. But for the complete decolourization the renewal of dialysing water was needed several times. The case with atmosphere of carbon dioxide was, however, quite different and the colour faded completely after a few hours in the first dialysis.

The addition of 5 cc. of hydrosulphuretted water to the dialysate in the last case produced a brown colouration, while each dialysate obtained in the medium of oxygen, hydrogen, air free from carbon dioxide and ordinary air remained almost colourless in the same treatment. It is highly probable that the copper-Yemorisol, though it had been prepared by electric discharge of metallic copper was readily oxided to copper oxide in contact with the ordinary air and partly dissolved into solution also as a copper carbonate, both acting bactericidally.

This consideration was ascertained also with the electrophoresis test which showed that the copper-Yemorisol was positively charged.

As to the phenomenon that the dialysate obtained in the medium of carbon dioxide contained a greater amount of copper compound but was far inferior to the other in its bactericidal power, the following two factors may be accounted for; 1. a diminution in the concentration of copper ion by the production of copper bicarbonate or 2. an influence of increas-

ed acidity in the dialysing medium by the saturation with carbon dioxide. To solve this question the following tests were made.

VI. Reactivation of Bactericidal Power of Dialysate Obtained in Atmosphere of Carbondioxide by Removal of Surplus Carbon Dioxide.

The dialysate obtained in the medium of carbon dioxide was bubbled with ordinary air for about 12 hours and its germicidal activity was compared with the one obtained in the ordinary air.

TABLE IV.

Number of colonies after treatment of bacteria with different dialysates.

Dialysate.		Duration of exposure.				
		15′	30′	45′	60′	90′
A		1300	860	310	240	110
B	before bubbling	∞	∞	∞	1600	980
	after bubbling	680	500	130	80	38

A. was obtained from copper-Yemorisol in the ordinary air.
B. was obtained from copper-Yemorisol in the carbon dioxide atmosphere.

As can be recognized from the above table the dialysate obtained in the carbon dioxide atmosphere had not only recovered its bactericidal activity on bubbling with air but acted rather more strongly than the others.

The cause of this result must be based upon an increase of copper ion in the dialysate by decomposition of copper bicarbonate on removal of an excess of carbon dioxide. Also, on bubbling with air the green colour of the solution appears.

VII. Influence of Acidity on the Bactericidal Action of the Dialysate.

N/10 acetic or hydrochloric acid and N/10 sodium hydroxide solution were diluted at the geometrical ratio of 3

from 1 to 729 times. 2 cc. of each dilution were added to 10 cc. of the dialysate obtained in the ordinary air. On comparing the bactericidal action it was found out just as expected that their action was extremely reduced at a definite concentration of hydrogen ion, that is, somewhat at pH=5.5, completely at pH=4.9 or below. On the alkali side also at pH=7.7 or above the action of both dialysate and copper-Yemorisol was perfectly inhibited

TABLE V.

Number of colonies, when bacteria were treated with the mixture of 10 cc. of dialysates and 2 cc. of acid solution.

Minutes.	Dilution of N/10 acid solution.					
	3^3	3^4	3^5	3^6	3^7	H_2O
15′	∞	∞	∞	1500	1120	1000
30′	∞	∞	∞	132	140	169
45′	∞	∞	1120	9		4
60′	∞	∞	800	—	—	—
pH	—	4.9	5.5	6.5	6.6	6.6

Number of colonies, when bacteria were treated with the mixture of 10 cc. of dialysates and 2 cc. of alkali solution.

Minutes.	Dilution of N/10 alkali solution.					
	3^3	3^4	3^5	3^6	3^7	H_2O
15′	∞	∞	∞	730	800	660
30′	∞	∞	1800	140	150	120
45′	∞	∞	400	32	30	16
60′	∞	∞	50	—	8	—
pH	—	9.1	7.1	6.8	6.7	6.7

Number of colonies, when bacteria were treated with the mixture of 10 cc. of copper-Yemorisol and 2 cc. of alkali solution.

Minutes.	Dilution of N/10 alkali solution.					
	3^3	3^4	3^5	3^6	3^7	H_2O
15′	∞	∞	∞	∞	∞	∞
30′	∞	∞	2000	570	560	600
45′	∞	∞	640	138	120	142
60′	∞	∞	150	28	31	38
90′	∞	∞	72	12	13	17
120′	∞	∞	11	5	—	4
pH	—	7.7	7.1	6.8	6.8	6.8

The reduction of bactericidal action of the acid side may be ascribed to an increased hydrogen ion concentration. As to the influence of acidity upon the bactericidal action of heavy metal salts the author made another series of experiments* and many interesting results were obtained. According to these results the value of pH which inhibits the action seems to be variable according either to the kind of metal salt or to its concentration.

It seems to me, therefore, that the diminution of bactericidal activity of copper carbonate solution with excess of carbon dioxide may be due partly to the scarce amount of copper ion in the solution and partly to the increase of acidity caused by the presence of carbon dioxide.

The reduction of the action on the alkali side might be caused by the diminution of the amount of copper ion due either to the conversion of copper carbonate to copper oxide or to the inhibition of dissociation of copper oxide by increased hydroxyl ion in the solution. The electrophoresis test reveals also the fact that on addition of alkali to a certain point the positively charged particles of copper-Yemorisol are converted to negatively charged ones. This circumstance may naturally contribute to the reduction of activity.

* The result will be reported in the next communication.

VIII. Reduction of Copper-Yemorisol.

To 10 cc. of 3 times diluted copper-Yemorisol placed in a flask of about 25 cc. capacity were added 4 cc. of N/10 hydrazin hydrochloride and 5 cc. of N/10 sodium hydroxide solution, then heated on the boiling water bath stirring the mixture well at intervals for about 10 minutes. Through this treatment the colour of the mixture was changed gradually to citron yellow, yellowish grey and at the end of reduction it became just transparent deep red. 10 cc. of such a solution were dialysed for 24 hours in an atmosphere of hydrogen gas against distilled water of 100 cc., which was previously vigorously boiled for about 5 minutes bubbling with hydrogen gas and cooled to room temperature under the air of the same gas. The similar process had been repeated three times, and its dialysate was found almost free from chloride. The electrical charge of the particles in the solution was proved as negative by means of electrophoresis test performed under the layer of liquid paraffin.

The solution thus obtained is extremely labile against oxygen or ordinary air, and on stirring in the air it changes almost momentarily into a dark blue solution and it returned to original colouration of the untreated copper-Yemorisol after several hours. From these observations there is no doubt that this red product represents a true colloidal solution of metallic copper. I have, therefore, worked out the following experiments with this solution.

IX. Bactericidal Power of the Dialysate of Red Solution.

Four dialysates were obtained by 24 hours' dialysis of 10 cc. of the red solution against distilled water under different gases—hydrogen, oxygen, air free from carbon dioxide and the ordinary air. Of course the red solution had been previously dialysed as noted above. As a control, another dialysate was obtained by dialysis of 10 cc. of copper-Yemorisol of the same concentration in the ordinary air. On making a com-

parative test of bactericidal power with every 10 cc. of those dialysates the result came out just as expected in the beginning but differed obviously from the result obtained with the dialysates of copper-Yemorisol which was noted in the third experiment.

TABLE VI.

Number of colonies after treatment of bacteria with the dialysates obtained from both red solution and Yemorisol under the atmosphere of different gases.

Duration of exposure.	Yemorisol.	Red solution.			
	ord. air.	hydrogen.	oxygen.	air-CO_2..	ordinary air.
10′	∞	∞	∞	∞	∞
30′	∞	∞	∞	∞	∞
45′	2000	∞	∞	∞	1700
60′	500	∞	∞	∞	260
60′	58	∞	∞	∞	23
120′	19	∞	∞	∞	—

As can be seen in the above table, the dialysate obtained by dialysis of the red solution in the medium of hydrogen was almost inactive, while the other three obtained in the medium of oxygen, air free from carbon dioxide and the ordinary air exerted a recognizable bactericidal action. Among them the dialysate obtained in the ordinary air was not only most effective in the action but it acted also as strongly as the one obtained from the untreated copper-Yemorisol.

These results suggest to us an assumption that on exposure of the colloidal solution of metallic copper to oxygen, a copper oxide is formed. As the solubility of the latter is very poor, copper ion does not exist in the solution to a sufficient amount to kill bacteria within a limited time. If, however, there is carbon dioxide present together with oxygen, such as in the ordinary air, the copper oxide particle formed is further affected by carbon dioxide, and converted into a copper carbonate which is more soluble than copper oxide. For this reason the dialysate obtained in the ordinary air exceeds others in its content of copper ion, and acts at most bactericidally.

X. Bactericidal Action of the Pure Metallic Colloidal Solution.

According to the above result hydrogen is naturally quite inefficient for the production of copper ion from colloidal copper particles. If the colloidal particle of metallic copper itself plays any part in the germicidal action of copper hydrosol, it could be expected that the copper sol would remain active even after the removal of the ionic copper from the solution by repeated dialysis in the medium of hydrogen. But in reality this is not the case, as will be seen in the following experiment.

10 cc. of the red solution were dialysed for 12 hours against distilled water freed from carbon dioxide by boiling and cooled under atmosphere of hydrogen. Such a dialysing process was repeated 4 times always in contact with hydrogen gas and a pure copper sol was obtained. On the other hand, every 10 cc. of the similar red solution, purified as mentioned above, were allowed to stand for 12 hours in the medium of oxygen or the ordinary air. The colour of the solution changed in the former case into deep blue, while that of the latter changed into greenish blue.

The bactericidal action of these 3 colloidal solutions was tested, keeping each solution always in contact with its respective gas.

TABLE VII.

Number of colonies when bacteria were treated with copper sols exposed to different gases.

Duration of exposure.	Ordinary air.	Oxygen.	Hydrogen.
15	1600	∞	∞
30	120	520	∞
45	9	260	∞
60	—	120	∞
90	—	20	∞
120	—	—	∞
pH	6.4	6.7	7.1

The greenish blue solution obtained in the air destroyed all bacteria within 60 minutes and the blue one obtained in the atmosphere of oxygen after 120 minutes, whereas the red one kept under hydrogen showed no indication of bactericidal activity even after 120 minutes.

From this experiment it becomes clear that the colloidal particle of pure metallic copper does not directly concern the germicidal action. In contact with oxygen, however, colloidal metallic copper is easily oxided to copper oxide, which exerts, in the form of copper ion, the germicidal action.

As owing to its poor solubility copper oxide can not send out a larger amount of copper ion into the solution, its dialysate does not exert the bactericidal activity to any great extent. The colloidal solution, however, acts more effectively, when brought into direct contact with bacteria. This might be referred to the mutual precipitation which takes place between negatively charged bacteria and positive copper oxide particles.

On exposure to the air the colloidal copper is not only oxided to copper oxide but it is further dissolved as a copper carbonate increasing the amount of the copper ion in the solution. As a consequence such a solution acquires strongest germicidal action.

XI. Conclusion.

1. The germicidal action of copper-Yemorisol rests only upon the copper ion ionized from both copper oxide and carbonate produced from colloidal metallic particles by the presence of atmospheric gases. Colloidal particles of pure metallic copper have not direct bearing on this action.

2. The bactericidal action of the dialysate obtained by dialysis of copper-Yemorisol is strongly affected by addition of acid solution and at a definite concentration of hydrogen ion the action is almost completely inhibited.

3. All mechanism which reduces a dissociation of copper carbonate, such as saturation with carbon dioxide, addition of

alkali solution or hydrosulphuretted water etc. annihilates the germicidal action of the dialysate obtained from copper-Yemorisol.

4. Colloidal solution of metallic copper, whether it is prepared by electrical or by chemical method, is oxided quite rapidly to copper oxide sol on contact with oxygen. The presence of carbon dioxide in the atmosphere induces further the production of copper carbonate, which is more soluble than copper oxide. These steps of change can be followed by observing the variation of the colour of colloidal solution as well as by determining the germicidal activity of the corresponding solution.

In conclusion the auther desires to acknowledge the receipt of kind instruction from Prof. S. Kakiuchi, the director of our institute.

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ON FATTY ACIDS OBTAINED FROM CEPHALIN. COMPOUNDS OF β-AMINOETHYL ALCOHOL WITH SATURATED AND UNSATURATED FATTY ACIDS.

By

RYOICHI KOGANEI.

(From the Biochemical Laboratory, Institute of Medical Chemistry, Tokyo Imperial University. Director: Prof. S. Kakiuchi.)

(Received for publication, February 1, 1923.)

During our effort to obtain pure fatty acids by hydrolysing cephalin, we met with two facts, which arrested our attention. The first is; the yields of fatty acid, when purified by the method described by Mottram (1910), were quite variable, and the second is; the fatty acids or their soaps obtained on hydrolysis invariably contained a small amount of nitrogen (see this journal, vol. II, p. 499).

It is generally understood that the contaminated nitrogen is due to aminoethyl alcohol (Trier, 1911, 1913; Baumann 1913; Renall, 1913), the significance of which, however. has been not at all clear.

To find out to what reason this fact is due, we attempted to purify these fatty acids by various methods and follow the change in their nitrogen contents.

Experiment I.

Different amounts of fatty acid, prepared by saponification of cephalin by means of sodium ethylate were dissolved separately in each 150 cc. of alkaline 50 per cent alcohol in three separating funnels, acidified and extracted three times by means of petroleum ether. The amount of fatty acid before and after the purification together with its nitrogen content is shown in the following table.

TABLE I.

In the beginning.		After the purification.		Loss.
Amount.	N-content.	Amount.	N-content.	
gm.	*per cent.*	*gm.*	*per cent.*	*gm.*
0.319	—	0.199	—	0.120
1.150	0.93	0.991	0.79 %	0.159
4.045	1.20	3.836	0.93 %	0.204

It can be seen from the table that there is always the same loss of nitrogen containing fatty acid, when the latter is purified by the method of Mottram, and also that thereby the nitrogen contamination is not reduced considerably.

Experiment II.

To remove the nitrogenous impurities from phospholipins the washing of their chloroform solution with NaCl solution is recommended (Sano, 1922). We have, therefore, applied this method to the fatty acid above mentioned to see whether it can be freed from the contaminated nitrogen.

0.3836 gm. of fatty acid obtained from cephalin was dissolved in 50 cc. chloroform and washed with the same volume of 1 per cent NaCl solution several times. After the evaporation of chloroform, 0.3158 gm. of fatty acid was obtained in the dry state. Nitrogen and phosphorus of the fatty acid before and after the washing were found to be as follows:

TABLE II.

	Nitrogen content.	Phosphorus content.
Before washing.	0.86 %	0.28 %
After washing.	0.70 %	0.15 %

Nitrogen impurity in this case seems, therefore, not to be removed by this treatment.

Experiment III.

In this experiment the purification of fatty acid by the treatment of lead acetate was tried and change in the nitrogen content was followed up.

By saponification of 2.5143 gm. of cephalin 0.9613 gm. of fatty acid (yield 38.2%) was obtained, which was taken up in hot alcohol and neutralized with alcoholic potassium hydroxide. To this soap solution an excess of watery solution of lead acetate was added to precipitate the fatty acid, and filtered.

The filtrate was concentrated, neutralized and again precipitated with lead acetate. These procedures were repeated till no more precipitation occurred. All the precipitate was combined and dried. To isolate the ether soluble lead salt quantitatively the precipitate was then triturated with ether several times, always renewing the ether and filtered (Oudemans, 1866).

a) The ether-insoluble residue was then extracted with 50 cc. of 3 per cent alcoholic hydrochloric acid. From this alcoholic solution, after the addition of 50 cc. of water, fatty acid was separated by the method of Mottram, and 0.3599 gm. of stearic acid was obtained.

b) From the ether-soluble portion of lead salt 0.054 gm. of light yellowish oil could be separated by the same method. This portion consisted of oleic acid (Levene and Rolf, 1922).

Nitrogen content in each of these fatty acids was found to be as follows:

	Nitrogen content
Fatty acid before separation	0.83 %
Stearic acid	0.70 %
Oleic acid	0.27 %

It may be concluded from this experiment that the fatty acid is quite difficult to get rid of from the contaminated

nitrogen even when it is precipitated as lead salt, and that these fatty acids, especially oleic acid, were very poorly precipitated from the alkaline soap solution by lead acetate in the presence of aminoethyl alcohol. From the combined filtrate of alcoholic soap solution we could recover almost the same amount of lost fatty acid, which did not solidify in entire dryness.

Summarizing the foregoing result it can be stated that the fatty acid prepared by saponification of cephalin, was contaminated by the nitrogenous impurities which were quite difficult to remove by purification. As it seemed to be obvious that this nitrogenous impurity is nothing other than β-aminoethyl alcohol, this same procedure of purification was conducted with the mixture of aminoethyl alcohol and fatty acids.

Extraction and Precipitation of Fatty Acid by the Presence of Aminoethyl Alcohol.

(1) 1.2228 gm. of oleic acid, after conversion to its potash soap, was dissolved in 50 cc. alcohol and 1 cc. of aminoethyl alcohol was added, which was prepared by the redistillation of the sample referred to by Kahlbaum; it boiled at 160°–162° C. (Wurtz, 1860). The mixture was then acidified and extracted three times with petroleum ether. The amount of recovered fatty acid and its nitrogen content was found to be as follows:

Yield of fatty acid, 0.195 gm. (16.13 %)
Nitrogen content, 0.905 %

(2) A certain amount of oleic or palmitic acid and 0.5 cc. of aminoethyl alcohol were dissolved in 20 cc. of alcohol, and after neutralization, precipitated by addition of 20 cc. of saturated watery solution of lead acetate.

The precipitate in each case was treated with three per cent HCl alcohol, from which corresponding fatty acid was separated by the method of Mottram. The result is shown in the following table:

TABLE III.

Nature of fatty acid.	Amount used.	Amount recovered.	
		absolute.	percentage.
	gm.	*gm.*	
Oleic acid (Kahlbaum).	0.8458	0.0115	13.0
Palmitic acid (Merck).	1.3378	0.3418	25.25

It can be seen from the table that in case of contamination of aminoethyl alcohol fatty acids are precipitated with difficulty from lead acetate in alcoholic solution. Oleic acid recovered from such lead precipitate was found to contain a considerable amount of nitrogen, it being about 3.8 % in the case above mentioned.

Compounds of Aminoethyl Alcohol and Fatty Acid.

Exactly 2 gm. of stearic acid (Merck, puriss) or oleic acid (Kahlbaum) were mixed separately in a weighed beaker with 1 cc. of aminoethyl alcohol. It may be remarked in passing that by mixing oleic acid with aminoethyl alcohol there was a fair production of heat observable.

20 cc. of absolute alcohol were then added to each beaker and the mixture was heated on the water bath to complete the combination, until the alcohol was evaporated off.

The remaining product presented itself as a light yellowish substance, and when dried at about 90° C. at reduced pressure (15 mm.) lost its weight gradually, till it reached a constant weight.

TABLE IV.

Duration of drying. *hours*	6	8	10	12
Weight of 2 gm. stearic acid + 1 cc. aminoethyl alcohol.	2.6918 *gm.*	2.4868 *gm.*	2.4493 *gm.*	2.4400 *gm.*
Weight of 2 gm. oleic acid + 1 cc. aminoethyl alcohol.	2.7197 *gm.*	2.4500 *gm.*	2.3697 *gm.*	—

If we presume that there occurred an equimolecular combination between fatty acid and aminoethyl alcohol the final weight of the mixture may be as 2.43 gm. (stearate) and 2.432 gm. (oleate) respectively.

With regard to their physical properties, each of these substances distinguished itself entirely from original components, and could easily be recognized as a new compound. It is for example fairly soluble in ether, while aminoethyl alcohol is insoluble in ether, and readily soluble in water, whereas fatty acid does not dissolve in water. Its watery solution is quite viscous, foams very easily and shows a thick opalescence, which closely resembles that of alkaline soap solution. 1 % oleate solution remains homogenous for a few days, whereas the stearate was decomposed undergoing hydrolysis and the stearic acid begins to separate out as glittering crystals. The reaction of these watery solutions is strongly alkaline.

So far as my knowledge goes, there is no mention made in the literature about these compounds, colamin (ethanolamin) stearate and oleate.

Extraction of Fatty Acids from Colamin Stearate and Oleate on Different Acidity.

Each of 0.243 gm. of stearate and 0.244 gm. of oleate was separately dissolved in 40 cc. of 50 % alcohol and extracted with 50, 25 and 25 cc. of petroleum ether successively on addition of hydrochloric acid at different concentrations.

TABLE V.

Molar concentration of HCl added.	0	0.5	1
Recovered from 0.243 gm. stearate.	0.1046 *gm.*	0.2020 *gm.*	0.2270 *gm.*
Recovered from 0,244 gm. oleate.	0.0791 *gm.*	0.2121 *gm.*	0.2286 *gm.*

As the original materials (0.243 and 0.244 gm.) contained exactly 0.2 gm. of stearic and oleic acid, the extracts obtained by the addition of 1 mol of HCl were of course not pure fatty acids and probably mixed with aminoethyl alcohol, which could be easily demonstrated from the fact that the extract contained a fair amount of nitrogen.

For comparative study the solution of 0.0433 mg. of aminoethyl alcohol in 50 per cent ethyl alcohol was acidified with 0.026 gm. of HCl and extracted by means of petroleum ether. Only 0.099 mg. nitrogen (i. e. 0.43 mg. aminoethyl alcohol) could be extracted by this method. Aminoethyl alcohol dissolves thorefore into petroleum ether more easily in presence of free fatty acid, and this is the reason why the obtained amount of fatty acid exceeded the quantity taken.

The Question of the Acid-resistance of Colamin Stearate and Oleate.

(I) 0.1215 gm. of stearate dissolved in 20 cc. of 50 % alcohol, was placed into each of five small separating funnels. At the lapse of varying intervals after the addition of 0,026 mg. HCl (double quantity of calculated amount to free the fatty acid) one after another was extracted with 50 cc. of petroleum ether, and the amount of extracted fatty acid is shown in the following table.

TABLE VI.

Time interval.	Amount of stearic acid recovered.	Nitrogen content.
minutes	*gm.*	%
1	0.0957	6.346
5	0.0976	—
15	0.0991	—
30	0.0985	—
60	0.0993	0.180

(II) The same experiment was repeated with 0,122 gm. of colamin oleate and its result is shown in the following table.

TABLE VII.

Time interval.	Amount of oleic acid recovered.	Nitrogen content.
minutes.	*gm.*	%
1	0.0653	0.405
5	0.0663	—
15	0.0665	—
30	0.0796	—
60	0.0728	0.355

As the table shows, the amount of recovered fatty acid was larger, the later the extraction was conducted. At the same time it is noticeable that the extracted fatty acid contained always nitrogen, the amount of which tends to decrease with the lapse of time after treatment with HCl, this change being more eclatant in the case of colamin stearate. This indicates the fact that the colamin-fatty acid compound is not easily dissociated into its components by the double amount of HCl to replace the fatty acid, and therefore, a certain amount of fatty acid remains in the alcohol-water layer, while, on the other hand, colamin is contained in greater quantity than corresponds with its solubility in petroleum ether. This is more especially so in the case of oleic acid compound.

Estimation of Aminonitrogen in Colamin Stearate and Oleate by the Method of Van Slyke.

(I) Estimated by the method of Van Slyke's amino-nitrogen determination, (1912, 1913) 0.0245 gm. of stearate yields for three analyses 1.63, 1.58 and 1.59 cc. of nitrogen (average 1.60 cc.) at 20° C. and 758.7 mm. i. e. 0.915 mg. It corresponds to 92.2 % of the calculated amount (0.995 mg.) of amino nitrogen.

(II) Yields of nitrogen from 0.0273 mg. oleate by the same method are for three analyses 1.34, 1.35 and 1.39 cc. (average 1.36 cc. i. e. 0.766 mg.) at 19.5° C. and 758.7 mm. It corresponds only to 79.2 per cent of calculated amount (0.968 mg.).

These results suggest to us that colamin oleate is with more difficulty dissociable at the acidity prevalent in the method than colamin stearate according to its more acidic character.

That the dissociation of colamin oleate, however, becomes complete by the prolongation of the shaking can be shown in the following experiments.

0.5 gm. of colamin oleate was dissolved in 25 cc. of water, and in each 2 cc. of it amino nitrogen was determined at various durations of shaking. Room temperature was 15° C., and the glacial acetic acid was exactly 3 cc. in each case.

TABLE VIII.

No. of exp.	Duration of shaking.	Amount of Amino-N.
	minutes	*mg.*
1	3	1.395
2	5	1.535
3	7	1.602
4	8	1.631
5	10	1.631

At the same time the amount of amino nitrogen in 2 cc. of 0.19 % colamin solution (its nitrogen content being 1.62 mg. by Kjeldahl's method) was estimated, changing the duration of shaking at the same room temperature.

TABLE IX.

No. of exp.	Duration of shaking.	Amount of Amino-N.
	minutes	*mg.*
1	3	1.483
2	4	1.543
3	5	1.605
4	6	1.620
5	8	1.630

From the above tables it is obvious that for the completion of reaction about 8 minutes was needed in the case of colamin oleate, while in the cases of pure colamin solution the reaction is ended within 6 minutes as is shown by Baumann (1913) and Renall (1913) in their studies on cephalin.

Experiments to Prove why the Fatty Acids Extracted by the Method of Mottram are Contaminated with Small but Persistent Amount of Nitrogen Even After Repeated Purifications.

As it become known that the fatty acid forms a salt with aminoethyl alcohol, which is soluble both in watery (and alcoholic) solution and ether (and petroleum ether), the extraction of fatty acid with petroleum ether would meet with some kinds of disturbances. The fatty acid might for example be partly retained in the watery solution, while, on the other hand, colamin could dissolve in petroleum ether, especially when the amount of fatty acid is large. To test this relation by the experiment the following step was taken.

0.0433 gm. of aminoethyl alcohol (nitrogen content: 9.94 mg.) dissolved in 50 cc. of 50 per cent alcohol, was shaken in a separating funnel with 50 cc. of petroleum ether, which contained various amounts of oleic acid.

After the mixture had been well shaken and kept over night, hydrochloric acid was added in sufficient quantity (0.026 gm.), and the shaking was repeated vigorously for thirty seconds. The supernatant petroleum ether was separated quantitively from the lower alcohol water layer, in which total amounts of residue and nitrogen content were determined. The results are shown in the following table.

TABLE X.

No. of experim.	Amount of aminoethyl alcohol and its nitrogen content.	Amount of oleic acid contained in petroleum ether.		Amount of substance dissolved in lower alcohol-water layer.	
		Before the experiment	After the experiment	Total amount	Nitrogen
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
1.	0.0433 (9.94 mg N)	0	0	0.0676 (colamin chloride)	9.96
2.	,,	0.2	0.1930*	0.0695	9.21
3.	,,	0.5	0.4918	0.0737	9.12
4.	,,	1.0	0.9814	0.0732	9.07
5.	,,	2.0	0.9540	0.0757	8.50

* In this oleic acid nitrogen was found to the amount 0.12–0.4 per cent.

Judging from the result shown in the table, in the presence of colamin the fatty acid can not be extracted completely by petroleum ether, and at the same time the fatty acid is contaminated by colamin to some extent. It is, therefore, quite obvious that the fatty acids obtained by the hydrolysis of cephalin are hardly to be freed from nitrogen. And it is also worthy of note that the determination of fat in tissue rich in cephalin has to meet with the unavoidability of error when carelessly conducted.

The affinity of fatty acid to aminoethyl alcohol naturally varies according the nature of fatty acid, the oleic acid compound, for instance, being much more stable than that of stearic acid. The aminoethyl alcohol forms, however, the most stable salt with inorganic acids, and if such a solution were shaken with petroleum ether containing fatty acid, it is obvious that the salt would resist decomposition and remain within the watery solution.

SUMMARY.

The aminoethyl alcohol forms with fatty acid a compound, which is not promptly dissociated even by the presence of inorganic acid and dissolves into the organic solvents, such as ether or petroleum ether, especially when a large quantity of free fatty acid is present, contaminating thus the fatty acids with nitrogenous impurity in its turn.

That the fatty acid obtained by hydrolysis of cephalin invariably retained a small amount of nitrogen, is thus easily explanable, and it might be well noted that the fat-determination of any cephalin containing organs will be affected by such a circumstance.

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ON THE EFFECT OF CALCIUM OXIDE AND CALCIUM CARBONATE UPON THE DECOMPOSITION OF SOY-BEAN CAKE AND HERRING CAKE IN TWO DIFFERENT SOILS.

By

KOJI MIYAKE and KOJI NAKAMURA.

(From the Institute of Agricultural Chemistry of Hokkaido Imperial University, Sapporo.)

(Received for publication, May 4, 1923)

Introduction.

With regard to the effect of the calcium oxide and calcium carbonate upon the decomposition of organic matter in soil, two opposite opinions exist at present; one is that lime interrupts the decomposition of organic matter. This is the theory of Wollney, E. (1897), Kossowitsch, P. and Trejokow, J. (1902), and others. The other view is that lime stimulates the decomposition of organic matter in soil, and Ramman, E. (1911), Lemmermann, O., Aso, K., Fischer, H., and Fresenius, L. (1911), Neller, J. R. (1918), and others support this theory.

On the other hand, there are many investigators who studied the effect of calcium oxide and calcium carbonate upon the ammonification and the nitrification of organic matter in soil, such as Lemmermann, O. and Fischer, H. (1911), Stephenson, R. E. (1918) (1921), Lipman, J. G. (1920), Neller, J. R. (1920), and others. And they all agree that the nitrification was stimulated very much by the addition of calcium oxide or calcium carbonate, while the ammonification was depressed.

Notwithstanding this great number of observers who investigated the decomposition as well as the ammonification and the nitrification of organic matter, there are very few

who have tried to find the correlation between the decomposition of the carbon compound and the nitrogen compound of organic matter. As far as is known to us, only Neller, J. R. (1918), and Gainey, P. L. (1919) have studied this subject. According to them the amount of the ammonified nitrogen increases or decreases parallel to the amount of CO_2 gas which is produced by the decomposition of the carbon compound of organic matter.

Thus, in spite of these numerous researches concerning the effect of calcium oxide and calcium carbonate upon the decomposition of organic matter, results do not agree. On the correlation between the decomposition of the carbon compound and the nitrogen compound of organic matter, there exist only a few studies.

Thinking, therefore, that this problem can only be solved by further investigations, we undertook the following experiments with soy-bean cake and herring cake which are used widely in Japan as a source of nitrogen, having three objects in mind, viz.

1. What are the effects of calcium oxide and calcium carbonate upon the decomposition of soy-bean cake and herring cake?

2. What effect have calcium oxide and calcium carbonate upon the ammonification and the nitrification of soy-bean cake and herring cake?

3. What correlation exists between the decomposition of the carbon compound and the ammonification of soy-bean cake and herring cake?

Samples.

As soil, loam and acid soil were used. The loam was taken from the experimental farm of our university, and the acid soil from Kutchan. These soils were dried in the air, and sifted with a 0.5 mm sieve. Some of their components and properties were determined as follows:—

	Loam.	Acid soil.
Organic carbon.	3.869 %	4.059 %
CO_2 in carbonate form.	0.055	0.033
Nitric nitrogen.	0.000	0.002
Ammonia nitrogen.	0.017	0.019
Water capacity.	75.0	83.3
Acidity (Daikubara's kalium chloride method).	6.75 cc.	58.5 cc.

The soy-bean cake and the herring cake were crushed by means of a mill, and sifted with a 0.5 mm. sieve. The constitutents of these cakes were as follows:—

	Soy-bean cake.	Herring cake.
Moisture.	14.49 %	10.91 %
Organic matter.	80.25	69.29
Organic carbon.	44.53	39.29
Total nitrogen.	7.54	10.91
Ash.	5.26	20.38

Pure powdered calcium oxide was heated in a porcelain basin. Then it was stored in a sulphuric acid dessicator, till it was used. It was assumed as pure CaO. And pure calcium carbonate of the following composition was used:—

CaO	53.02 %
CO_2	43.98 %

Experiments.

10 gm. of loam or acid soil, 0.05 gm. of soy-bean cake or herring cake, and 0.1 gm., 0.05 gm. or 0.01 gm. of CaO or $CaCO_3$ were taken into a porcelain basin, and mixed very well, adding 4.5 cc. of water to loam, likewise 4.9 cc. of water were added to the acid soil (that is 60 % of the water capacity of the soil). Then the soil was put in an Erlenmeyer flask, corked and waxed. The cork had two bent glass tubes and these had gum tubes attached which were closed with glass rods. Then the flask was put in the thermostat at 30° C.

The experiments lasted 4 weeks, and at the end of every week the quantity of CO_2 gas which was produced by the decomposition of organic matter was estimated. At the end of 2 and 4 weeks the quantity of CO_2 which existed in the soil as carbonate was also estimated. At the end of 4 weeks the organic carbon which remained in the soil was also determined. In order to determine the quantity of CO_2 gas produced by the decomposition, the gas in the flask was introduced in 100 cc. of $Ba(OH)_2$ solution of known concentration through one of the glass tubes by replacing the gas in the flask with CO_2 free air through the other glass tube. When this was ended, the precipitates of $BaCO_3$ were filtered rapidly under due precaution against the combination of the barium with the CO_2 gas in the air. Then the filtrate was titrated with standard sulphuric acid, and the barium which had combined with CO_2 gas in the flask was determined and therefrom the amount of CO_2 calculated.

The carbonic acid, which existed in the soil as a carbonate, was decomposed by sulphuric acid, and the CO_2 gas produced absorbed in a kali bulb and its weight estimated. And this weight contained beside the amount of the carbonate which was produced by the decomposition of organic matter, also the amount of CO_2 in carbonate form in the soil, so that amount of CO_2 in carbonate form of organic origin was calculated by the following subtraction:—

1) In case when CaO was added:—

Estimated amount of CO_2 in carbonate form in sample after 2 or 4 weeks.

Amount of CO_2 in carbonate form in soil at the beginning of the experiment.

$-)$

Amount of CO_2 in carbonate form which was produced by the decomposition of organic matter after 2 or 4 weeks.

2) In case when $CaCO_3$ was added:—

Estimated amount of CO_2 in carbonate form in sample after 2 or 4 weeks.

Amount of CO_2 in carbonate form in soil at the beginning plus amount of CO_2 which is contained in $CaCO_3$.
$-)$
Amount of CO_2 in carbonate form which was produced from organic matter in 2 or 4 weeks.

For the estimation of organic carbon in soil, we used a modified method of Cameron and Brageale who had estimated the organic carbon using potassium bichromate and sulphuric acid. The organic carbon of the organic matter added, which was not decomposed in 4 weeks but remained in the soil, was calculated by the following subtraction:—

Estimated amount of organic carbon which exists in a sample after 4 weeks.
$-)$
Amount of organic carbon which exists in the soil at the beginning of the experiments.
Amount of organic carbon of organic matter which remined in the soil after 4 weeks.

This subtraction was carried out because we could assume that the soil organic carbon compound did not decompose during the period mentioned as it does not decompose as readily as that of organic matter added.

These results were collected in Tables 1, 2, 3 and 4.

TABLE I.

Soy-bean cake in loam.

	Amount of CO_2 gas produced. (*gm.*)				
	0–1 week.	1–2 weeks.	2–3 weeks.	3–4 weeks.	Total.
No lime.	0.0092	0.0164	0.0083	0.0062	0.0401
CaO 1.0 %	0.0121	0.0211	0.0062	0.0035	0.0429
CaO 0.5 %	0.0108	0.0169	0.0052	0.0039	0.0368
CaO 0.1 %	0.0089	0.0143	0.0052	0.0047	0.0331
$CaCO_3$ 1.0 %	0.0214	0.0285	0.0196	0.0094	0.0789
$CaCO_3$ 0.5 %	0.0134	0.0235	0.0075	0.0062	0.0506
$CaCO_3$ 0.1 %	0.0094	0.0151	0.0078	0.0036	0.0379

	Amount of CO_2 in carbonate form in soil (*gm.*)		Amount of CO_2 in gas and carbonate form (*gm.*)
	End of 2 weeks	End of 4 weeks	End of 4 weeks
No lime.	0.0051	0.0052	0.0452
CaO 1.0 %	0.0153	0.0155	0.0584
CaO 0.5 %	0.0127	0.0130	0.0498
CaO 0.1 %	0.0107	0.0105	0.0436
$CaCO_3$ 1.0 %	—0.0238	—0.0235	0.0556
$CaCO_3$ 0.5 %	—0.0022	—0.0020	0.0486
$CaCO_3$ 0.1 %	0.0054	0.0054	0.0434

	Amount of organic carbon of soy-bean cake in soil. (*gm.*)		Percentage of decomposed organic carbon of soy-bean cake in soil.
	At the beginning	End of 4 weeks	In 4 weeks
No lime	0.0223	0.0104	53.36
CaO 1.0 %	0.0223	0.0066	70.40
CaO 0.5 %	0.0223	0.0087	60.98
CaO 0.1 %	0.0223	0.0105	52.92
$CaCO_3$ 1.0 %	0.0223	0.0073	67.26
$CaCO_3$ 0.5 %	0.0223	0.0093	58.30
$CaCO_3$ 0.1 %	0.0223	0.0106	52.46

TABLE II.

Herring cake in loam.

	Amount of CO_2 gas produced. (*gm.*)				
	0–1 week.	1–2 weeks.	2–3 weeks.	3–4 weeks.	Total.
No lime.	0.0118	0.0096	0.0059	0.0020	0.0293
CaO 1.0 %	0.0092	0.0120	0.0049	0.0019	0.0280
CaO 0.5 %	0.0132	0.0080	0.0033	0.0016	0.0261
CaO 0.1 %	0.0099	0.0075	0.0049	0.0023	0.0246
$CaCO_3$ 1.0 %	0.0206	0.0271	0.0107	0.0077	0.0661
$CaCO_3$ 0.5 %	0.0173	0.0118	0.0072	0.0043	0.0406
$CaCO_3$ 0.1 %	0.0104	0.0072	0.0065	0.0033	0.0274

	Amount of CO_2 in carbonate form in soil. (*gm.*)		Amount of CO_2 in gas and carbonate form. (*gm.*)
	End of 2 weeks.	End of 4 weeks.	End of 4 weeks.
No lime.	0.0073	0.0075	0.0368
CaO 1.0 %	0.0192	0.0190	0.0470
CaO 0.5 %	0.0168	0.0165	0.0426
CaO 0.1 %	0.0146	0.0143	0.0389
$CaCO_3$ 1.0 %	—0.0230	—0.0227	0.0434
$CaCO_3$ 0.5 %	—0.0005	—0.0003	0.0403
$CaCO_3$ 0.1 %	0.0098	0.0095	0.0367

	Amount of organic carbon of herring cake in soil. (*gm.*)		Percentage of decomposed organic carbon of herring cake in soil.
	At the beginning.	End of 4 weeks.	In 4 weeks.
No lime.	0.0196	0.0098	50.00
CaO 1.0 %	0.0196	0.0071	63.77
CaO 0.5 %	0.0196	0.0082	58.16
CaO 0.1 %	0.0196	0.0090	54.08
$CaCO_3$ 1.0 %	0.0196	0.0079	59.70
$CaCO_3$ 0.5 %	0.0196	0.0086	56.12
$CaCO_3$ 0.1 %	0.0196	0.0095	51.53

TABLE III.

Soy-bean cake in acid soil.

	Amount of CO_2 gas produced. (*gm.*)				
	0–1 week.	1–2 weeks.	2–3 weeks.	3–4 weeks.	Total.
No lime.	0.0053	0.0092	0.0088	0.0058	0.0291
CaO 1.0 %	0.0132	0.0163	0.0083	0.0051	0.0428
CaO 0.5 %	0.0092	0.0118	0.0096	0.0065	0.0371
CaO 0.1 %	0.0078	0.0107	0.0098	0.0069	0.0352
$CaCO_3$ 1.0 %	0.0221	0.0262	0.0185	0.0078	0.0746
$CaCO_3$ 0.5 %	0.0151	0.0214	0.0119	0.0081	0.0565
$CaCO_3$ 0.1 %	0.0097	0.0111	0.0088	0.0059	0.0355

	Amount of CO_2 in carbonate form in soil. (*gm.*)		Amount of CO_2 in gas and carbonate form. (*gm.*)
	End of 2 weeks.	End of 4 weeks.	End of 4 weeks.
No lime.	0.0055	0.0060	0.0351
CaO 1.0 %	0.0144	0.0140	0.0568
CaO 0.5 %	0.0099	0.0096	0.0467
CaO 0.1 %	0.0068	0.0068	0.0420
$CaCO_3$ 1.0 %	—0.0238	—0.0234	0.0512
$CaCO_3$ 0.5 %	—0.0100	—0.0094	0.0471
$CaCO_3$ 0.1 %	0.0029	0.0024	0.0379

	Amount of organic carbon of soy-bean cake in soil. (*gm.*)		Percentage of decomposed organic carbon of soy-bean cake in soil.
	At the beginning.	End of 4 weeks.	In 4 weeks.
No lime.	0.0223	0.0133	40.36
CaO 1.0 %	0.0223	0.0070	68.61
CaO 0.5 %	0.0223	0.0097	56.50
CaO 0.1 %	0.0223	0.0108	51.57
$CaCO_3$ 1.0 %	0.0223	0.0085	61.88
$CaCO_3$ 0.5 %	0.0223	0.0096	56.95
$CaCO_3$ 0.1 %	0.0223	0.0125	43.93

TABLE IV.

Herring cake in acid soil.

	Amount of CO_2 gas produced. (*gm.*)				
	0–1 week.	1–2 weeks.	2–3 weeks.	3–4 weeks.	Total.
No lime.	0.0119	0.0092	0.0050	0.0033	0.0294
CaO 1.0 %	0.0181	0.0146	0.0090	0.0021	0.0438
CaO 0.5 %	0.0142	0.0120	0.0079	0.0044	0.0385
CaO 0.1 %	0.0129	0.0081	0.0055	0.0041	0.0306
$CaCO_3$ 1.0 %	0.0288	0.0256	0.0145	0.0106	0.0795
$CaCO_3$ 0.5 %	0.0241	0.0183	0.0084	0.0059	0.0567
$CaCO_3$ 0.1 %	0.0146	0.0108	0.0059	0.0035	0.0348

	Amount of CO_2 in carbonate form in soil. (*gm.*)		Amount of CO_2 in gas and carbonate form. (*gm.*)
	End of 2 weeks.	End of 4 weeks.	End of 4 weeks.
No lime.	0.0063	0.0063	0.0357
CaO 1.0 %	0.0151	0.0157	0.0595
CaO 0.5 %	0.0105	0.0101	0.0486
CaO 0.1 %	0.0073	0.0072	0.0378
$CaCO_3$ 1.0 %	—0.0216	—0.0213	0.0582
$CaCO_3$ 0.5 %	—0.0091	—0.0088	0.0479
$CaCO_3$ 0.1 %	0.0018	0.0012	0.0360

	Amount of organic carbon of soy-bean cake in soil. (*gm.*)		Percentage of decomposed organic carbon of soy-bean cake in soil.
	At the beginning.	End of 4 weeks.	In 4 weeks.
No lime.	0.0196	0.0099	49.49
CaO 1.0 %	0.0196	0.0034	82.65
CaO 0.5 %	0.0196	0.0064	67.34
CaO 0.1 %	0.0196	0.0094	52.04
$CaCO_3$ 1.0 %	0.0196	0.0039	80.10
$CaCO_3$ 0.5 %	0.0196	0.0066	66.33
$CaCO_3$ 0.1 %	0.0196	0.0097	50.51

When the effect of CaO upon the decomposition of soy-bean cake in loam was observed, it could be seen that during the first period the addition of 1 % CaO stimulated its decomposition more than when lime was absent. In the second period the amount of CO_2 gas produced reached about twice the amount of that of the unlimed soil. In the third and fourth period the quantity of CO_2 gas produced was smaller than in the case of the unlimed soil; this took place because the amount of soy-bean cake itself and the amount of the easily decomposable constituents of soy-bean cake had decreased. The total quantity of CO_2 gas produced was not greatly increased by the addition of CaO, but the CO_2 which exists in the soil as a carbonate was very abundant, and at the end of 2 and 4 weeks it amounted to over 3 times the quantity of the unlimed soil. Therefore, the total quantity of CO_2 which was produced by the decomposition of soy-bean cake was considerably greater when lime was added, and the effect of CaO upon the decomposition of soy-bean cake was great. If all CaO added would combine with the CO_2, 0.1 gm. of CaO could combine with 0.07847 gm. of CO_2. But the result showed that only 0.0155 gm. CO_2 in carbonate form was produced. In explanation, we may assume that perhaps the soy-bean cake was decomposed producing organic acid, whereupon this organic acid would combine with the CaO forming an organic acid salt which was decomposed to carbonate. In a further step, this carbonate would be decomposed by the organic acid freeing its containing CO_2 in gas form. The salt of organic acid formed turned to carbonate again. The amount of organic acid produced would decrease parallel with the progress of the decomposition of the soy-bean cake, and at last, the remaining CaO would turn to carbonate. During this experiment, therefore, most of the CaO added would combine with the organic acid and a smaller part would combine with the CO_2 in the form of transitory carbonate. On account of this, the organic salt existed while the decom-

position was taking place. In this case, the amount of organic carbon decomposed was about 17 % more than in the case of the unlimed soil.

When 0.5 % of CaO was added, the quantity of CO_2 gas produced was, at the end of one week, larger than in the case of the unlimed soil, but less in the second, third or fourth period, and the total quantity for the whole period of this experiment, too, was smaller. However, when the amount of CO_2 existing in carbonate form in the soil was determined, it was found that at the end of 2 and 4 weeks this amount was over 2.4–2.5 times as much as that of the unlimed soil. Therefore, the total amount of CO_2 which was produced by the decomposition of soy-bean cake in gas and carbonate form was greater than that of the unlimed soil. If we judge solely from the point of view of CO_2 gas production, one might think that the addition of 0.5 % CaO interrupted the decomposition of the soy-bean cake. So if Wollney, E. (1897) is of opinion that the lime interrupts the decomposition of organic matter, he must have come to his conclusion by basing his deduction on the results of CO_2 gas production. Theoretically, 0.05 gm. of CaO could combine with 0.03925 gm. of CO_2, but the results showed less than this amount. As mentioned above, these results were attained because most of the CaO added would combine with the organic acid which was produced by the decomposition of soy-bean cake.

The effect of the addition of 0.1 % of CaO upon the decomposition of soy-bean cake was too slight to be observed by the production of CO_2 gas. But the effect upon the formation of carbonate in the soil was great, the quantity of carbonate being about twice as large as in the case of the unlimed soil, and larger than the theoretical amount of CO_2 which 0.01 gm. of CaO could combine. This might be due to the possibility that the carbonic acid, produced by the decomposition of soy-bean cake, may have become saturated

with the CaO (added in a small quantity) and subsequently may have formed a combination with the soil base. Therefore, in this case, the effect of CaO upon the decomposition of organic matter would be small. In fact, the percentage of the decomposed organic carbon of soy-bean cake was less than that of the unlimed soil. However, as it cannot be taken as probable that the addition of CaO interrupted the decomposition of organic matter, the unexpected results of this experiment may be due to an experimental error.

When the effect of 1% $CaCO_3$ upon the decomposition of soy-bean cake in loam was examined, it was found that at any period it increased considerably the production of CO_2 gas, the total amount of CO_2 gas produced being about twice the amount of that in the unlimed soil. But the quantity of carbonate was smaller than that of the unlimed soil. And judging from the fact that the quantity of CO_2 gas produced was greater in the beginning, we can assume that the $CaCO_3$ added must have been decomposed by the organic acid which was produced by the decomposition of soy-bean cake, setting the containing CO_2 free, whereupon the calcium may have combined with that acid. Therefore, the total quantity of the CO_2 gas produced only by the decomposition of soy-bean cake was not great comparatively. Compared with the effect of CaO, the action of $CaCO_3$ was inferior, and the percentage of the decomposed organic carbon was 3% less than in the case of CaO.

The same relation were seen when 0.5% of $CaCO_3$ was added. This salt was decomposed by the organic acid, produced by the decomposition of soy-bean cake, and its CO_2 was set free in gas form. The amount of CO_2 gas was also greater than in the case of the unlimed soil, but the amount which was produced exclusively from the soy-bean cake was not so large. Also in this case it was noticeable that the effect of $CaCO_3$ was inferior to that of CaO, the percentage of the decomposed organic carbon of soy-bean cake being 2.5% less.

The effect of 0.1 % $CaCO_3$ was also small. The total amount of CO_2 gas produced was smaller than in the case of the unlimed soil, the quantity of CO_2 in carbonate form, however, was greater. The total amount of CO_2 in gas and carbonate form was smaller. And the effect of 0.1 % $CaCO_3$ was also smaller than that of CaO, the percentage of the decomposed organic carbon of soy-bean cake being 0.5 % smaller. Thus, that the effect of $CaCO_3$ could not be determined might be an experimental error, and it might exist though it was small. Judging from the fact that the quantity of CO_2 gas produced was great in the beginning, we may assume that the $CaCO_3$ added was decomposed by the organic acid which was produced by the decomposition of soy-bean cake, thus freeing the containing CO_2 in gas form, whereupon the calcium would combine with this acid. Subsequently this salt of organic acid was decomposed in its turn, producing CO_2, so that it seems that the $CaCO_3$ added was not decomposed, but helped to increase the carbonate in the soil.

Next, the action of lime upon the decomposition of herring cake in loam was examined. The results showed that, in general, its effect was greater than in the case of the soy-bean cake, and that, irrespective of the quantity of lime used, it stimulated the decomposition of herring cake. The effect of CaO and $CaCO_3$ upon the production of CO_2 gas and carbonate in soil corresponds with the results obtained with the soy-bean cake, but as the herring cake was of animal origin and, therefore, rich in protein substance, the decomposition of it was fast and the production of CO_2 gas was very great during the initial period. However, its amount was highest in the second period, when 1 % of CaO or $CaCO_3$ was added. In this case also the effect of CaO was better than that of $CaCO_3$. When 1 %, 0.5 % and 0.1 % of CaO were added, the organic carbon of herring cake decomposed was respectively 13 %, 8 % and 4 % more than in the case of the unlimed soil. When 1 %, 0.5 % and 0.1 % of

$CaCO_3$ were added the organic carbon of herring cake decomposed was respectively 4 %, 2 % and 3 % smaller than when the same amount of CaO was added.

With respect to the effect of lime upon the decomposition of soy-bean cake and herring cake in acid soil, a similar action to that in the case of loam could be observed, and even the addition of 0.1 % of lime acted favorably. This was probably due to the fact that the lime acted favorably upon the growth and the activity of bacteria through a chemical and physical action tending to neutralise the soil acidity and thereby improve the soil texture and structure. Neller, J. R. (1920) reported that the oxidising power of the Sasafras-loam was reversely proportionate to its lime requirement, and this agrees with our acid soil experiments. As in the case of loam, the effect of CaO was greater than that of $CaCO_3$, and the effect of both CaO and $CaCO_3$ was greater in this soil than in the loam. When 1 %, 0.5 % and 0.1 % of CaO were added, the percentage of decomposed organic carbon was respectively 28 %, 16 % and 11 % more in the case of soy-bean cake, and 33%, 18% and 3% more in the case of herring cake than when lime was omitted. When 1 %, 0.5 % and 0.1 % of $CaCO_3$ were added, the decomposition of organic carbon was 7 %, 0.45 % (?) and 8 % less in the case of soy-bean cake and 2 %, 1 % and 3 % less in the case of herring cake compared with the case of CaO. (0.5 % $CaCO_3$ added produced better result than the same amount of CaO, but this might be an experimental error.)

As the effect of CaO was not greatly different from that of $CaCO_3$, these results, therefore, were averaged and the ratio between the percentage of organic carbon decomposed when lime was added and when lime was omitted calculated. It was as follows:—

In the case of soy-bean cake.

	No lime: 1 % lime	No lime: 0.5 % lime	No lime: 0.1 % lime
In loam	1: 1.3	1: 1.1	1: 1.0
In acid soil	1: 1.6	1: 1.4	1: 1.2

In the case of herring cake.

	No lime: 1 % lime	No lime: 0.5 % lime	No lime: 0.1 % lime
In loam	1: 1.3	1: 1.1	1: 1.0
In acid soil	1: 1.6	1: 1.3	1: 1.1

Concerning the effect of lime upon the decomposition of organic matter in soil, it can be said from the above result that the effect of lime differed only according to the kind of soil, but not according to the character of organic matter.

In short, the lime stimulates the decomposition of organic matter in soil, and for a certain soil and a certain kind of organic matter the optimum amount of lime would effect the greatest decomposition of organic matter. In this experiment, the addition of 1 % of lime gave a better result than the addition of 0.5 % or 0.1 %. This does not agree with the findings of Lemmermann, O. (1911), but this may be due to the difference in the experimental method and the samples used.

In another set of experiments, a mixture of soil, organic matter, lime and water was put in an Erlenmeyer flask and kept in the thermostat at 30° C. At the end of every week the ammonia nitrogen and the nitric nitrogen were estimated. To determine the ammonia nitrogen the magnesium oxide method was adopted, and the nitric nitrogen was estimated by the colorimetric method through addition of phenoldisulphonic acid. The amount of ammonified nitrogen which is shown in the table is the sum of the amount which was estimated as ammonia nitrogen and as nitric nitrogen, because the nitric nitrogen appears only by the oxidation of ammonia nitrogen, as was reported by Wollney, E. (1897), Stephensen, R. E. (1918), Fischer, H. (1911), Plummer, J. K. (1916), Stocklasa, J. (1912) and others.

These results are collected in Tables 5, 6, 7 and 8.

TABLE V.

Soy-bean cake in loam.

	Amount of ammonified nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.3328	0.4993	1.5815	1.8899
CaO 1.0 %	0.3132	0.6742	2.1256	2.[illegible]
CaO 0.5 %	0.3188	0.6578	1.3451	2.[illegible]
CaO 0.1 %	0.3189	0.4329	1.9063	1.92[illegible]
$CaCO_3$ 1.0 %	0.3256	0.8324	1.9063	3.4171
$CaCO_3$ 0.5 %	0.3258	0.6036	1.5824	2.5583
$CaCO_3$ 0.1 %	0.3595	0.4415	1.1534	2.3081

	Amount of nitric nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.0411	0.2737	1.3560	1.7967
CaO 1.0 %	0.0215	0.3164	1.9662	2.8069
CaO 0.5 %	0.0272	0.3661	1.1526	2.1763
CaO 0.1 %	0.0275	0.1413	0.7797	1.8306
$CaCO_3$ 1.0 %	0.0339	0.4746	1.7469	3.3900
$CaCO_3$ 0.5 %	0.0341	0.3119	1.3899	2.5312
$CaCO_3$ 0.1 %	0.0678	0.1498	0.8949	2.2148

TABLE VI.

Herring cake in loam.

	Amount of ammonified nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.3445	0.3787	1.2019	1.7255
CaO 1.0 %	0.3075	0.8189	1.5047	3.6205
CaO 0.5 %	0.3249	0.6985	1.2742	2.1967
CaO 0.1 %	0.3423	0.6542	1.0934	1.9261
$CaCO_3$ 1.0 %	0.3261	0.9127	2.3812	3.3493
$CaCO_3$ 0.5 %	0.3350	0.7053	1.8305	2.3323
$CaCO_3$ 0.1 %	0.3464	0.3787	1.0098	2.0943

	Amount of nitric nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.0528	0.2531	0.9763	1.5323
CaO 1.0 %	0.0158	0.4611	1.2792	3.5934
CaO 0.5 %	0.0333	0.4068	1.0486	2.1696
CaO 0.1 %	0.0506	0.3626	0.8678	1.8329
$CaCO_3$ 1.0 %	0.0344	0.6210	2.2218	3.3222
$CaCO_3$ 0.5 %	0.0433	0.4136	1.6046	2.3052
$CaCO_3$ 0.1 %	0.0547	0.2828	1.0842	2.0011

TABLE VII.

Soy-bean cake in acid soil.

	Amount of ammonified nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.5341	0.6145	0.7104	0.8911
CaO 1.0 %	0.8003	0.8454	1.0833	1.3042
CaO 0.5 %	0.7629	0.7856	0.9483	1.1226
CaO 0.1 %	0.6731	0.6843	0.8981	1.0711
$CaCO_3$ 1.0 %	0.7794	0.8725	1.2150	1.5212
$CaCO_3$ 0.5 %	0.7177	0.7928	1.1389	1.2437
$CaCO_3$ 0.1 %	0.6461	0.7223	1.0304	1.1199

	Amount of nitric nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.2034	0.2838	0.3797	0.6265
CaO 1.0 %	0.2373	0.3824	0.6765	1.0396
CaO 0.5 %	0.3661	0.3688	0.5515	0.7919
CaO 0.1 %	0.2763	0.2875	0.5012	0.7404
CaCO 1.0 %	0.3164	0.4095	0.8182	1.2566
CaCO 0.5 %	0.3209	0.3960	0.8082	0.9791
CaCO 0.1 %	0.2493	0.3255	0.6997	0.7892

TABLE VIII.

Herring cake in acid soil.

	Amount of ammonified nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.5364	0.6892	0.7965	0.8948
CaO 1.0 %	0.7161	0.8779	1.1392	1.5059
CaO 0.5 %	0.6341	0.7765	0.9031	1.1653
CaO 0.1 %	0.6074	0.7439	0.8742	1.1273
$CaCO_3$ 1.0 %	0.7206	0.8891	1.1392	1.5762
$CaCO_3$ 0.5 %	0.6517	0.8646	0.9370	1.2451
$CaCO_3$ 0.1 %	0.6490	0.8351	0.9045	1.1881

	Amount of nitric nitrogen. (*mg.*)			
	1 week.	2 weeks.	3 weeks.	4 weeks.
No lime.	0.2057	0.3254	0.3336	0.5641
CaO 1.0 %	0.2531	0.4149	0.7424	1.1752
CaO 0.5 %	0.2373	0.3797	0.5393	0.8346
CaO 0.1 %	0.2106	0.3471	0.4204	0.7966
$CaCO_3$ 1.0 %	0.2567	0.3930	0.7424	1.4780
$CaCO_3$ 0.5 %	0.2549	0.3685	0.5071	0.8482
$CaCO_3$ 0.1 %	0.2522	0.3390	0.4746	0.7912

The addition of CaO or $CaCO_3$ showed, at the initial stage of the experiment, a tendency to interrupt the ammonification in loam, if the amount of lime was large, and this tendency was clear when CaO was added. This small amount of ammonified nitrogen may be due to a considerable increase of the soil alkalinity caused by the addition of CaO, as Hoagland (1918) said that the initial effect of CaO was greatly to increase the OH ion concentration. However, the amount of the ammonified nitrogen became gradually greater and surpassed the corresponding quantity of the unlimed soil. When 1 %, 0.5 % and 0.1 % of CaO were added, the amount of the ammonified nitrogen was at the end of 4 weeks respectively 26 %, 8 % and 1 % more in the case of soy-bean cake, and 35 %, 9 % and 4 % more in the case of herring cake than when lime was omitted. The effect of $CaCO_3$ upon the ammonification of organic matter was greater than that of CaO. This fact stands in opposition to the results gained in an earlier experiment when the action of CaO upon the decomposition of the carbon compound of organic matter was examined and when it was found that it was greater than in the case of $CaCO_3$. And when 1 %, 0.5 % and 0.1 % $CaCO_3$ were added, the amount of ammonified nitrogen, at the end of 4 weeks, was respectively 41 %, 18 % and 11 % higher in the case of soy-bean cake, and 30 %, 11 % and 7 % higher in the case of herring cake than the corresponding figures for the unlimed soil indicated.

At the initial stage of the experiment the amount of the ammonified nitrogen in acid soil was greater than in the case of the unlimed soil. This result differed a little from the experiment with loam, but this may be due to the fact that, at the beginning of the experiment, the alkalinity of the soil caused by the addition of CaO was smaller in acid soil than that of loam owing to the neutralization of the acidity of the soil and it acted favorably upon the ammonification. The amount of the ammonified nitrogen was greater, at the

end of 3 or 4 weeks, than in the case of the unlimed soil, but smaller than the quantity which was ascertained in the experiment with the loam. The amount of the ammonified nitrogen of the soy-bean cake was respectively 11 %, 6 % and 5 % more than that of the unlimed soil when 1 %, 0.5 % and 0.1 % CaO were added; in the soil mixed with herring cake the amount of ammonified nitrogen was 11 %, 5 % and 4 % more, when 1 %, 0.5 % and 0.1 % CaO were added. The effect of $CaCO_3$ was also greater than that of CaO in this soil, and the amount of the ammonified nitrogen was 17 %, 9 % and 6 % more in the case of soy-bean cake, and 12 %, 6 % and 5 % more in the case of herring cake than that of the unlimed soil, when 1 %, 0.5 % and 0.1 % $CaCO_3$ were added respectively.

Next, we observed the action of lime upon the nitrification of soy-been cake or herring cake, and found that its effect was similar to its action upon the ammonification. We found that, in the case of loam, the nitrification was initially interrupted by the addition of lime, but in the later periods lime acted as a stimulant. In case of acid soil this stimulating influence begins at the start. The effect of lime upon the nitrification is related to the amount added, and the effect of $CaCO_3$ was greater than that of CaO. Plummer, J. K. (1916) reported that the amount of CO_2 gas produced indicated the amount of the nitrified nitrogen, and this may be true, because the amount of CO_2 gas produced as well as the quantity of nitrified nitrogen was great when $CaCO_3$ was added.

As the effect of CaO was not greatly different from that of $CaCO_3$, these results, therefore, were averaged, and then the ratio between the percentage of ammonified nitrogen and also of nitric nitrogen for limed and unlimed soil calculated as follows:—

Ammonification.

	In the case of soy-bean cake.		
	No lime: 1 % lime	No lime: 0.5 % lime	No lime: 0.1 % lime
In loam	1: 1.7	1: 1.3	1: 1.1
In acid soil	1: 1.6	1: 1.3	1: 1.2
	In the case of herring cake.		
	No lime: 1 % lime	No lime: 0.5 % lime	No lime: 0.1 % lime
In loam	1: 2.0	1: 1.3	1: 1.1
In acid soil	1: 1.7	1: 1.3	1: 1.2

Nitrification.

	In the case of soy-bean cake.		
	No lime: 1 % lime	No lime: 0.5 % lime	No lime: 0.1 % lime
In loam	1: 1.7	1: 1.3	1: 1.1
In acid soil	1: 1.8	1: 1.3	1: 1.1
	In the case of herring cake.		
	No lime: 1 % lime	No lime: 0.5 % lime	No lime: 0.1 % lime
In loam	1: 2.2	1: 1.4	1: 1.2
In acid soil	1: 2.3	1: 1.5	1: 1.4

According to these results, the effect of lime upon the ammonification differed from its effect upon the nitrification. Although the effect of lime upon the ammonification may be the same upon two kinds of organic matter, it may differ according to the kinds of soil. In the same way it seems probable that the effect of lime upon the nitrification is the same for different kinds of soil, but shows differencies according to the origin of organic matter.

In short the lime stimulates the ammonification and the nitrification of organic matter in soil, and its effect increases parallel with the quantity added, and the effect of CaO is smaller than that of $CaCO_3$. This fact and the fact that the effect of lime upon the decomposition of the carbon compound and upon the ammonification differed, according to the kind of soil, suggested to us the idea that there was the correlation between the decomposition of the carbon compound and the ammonification of organic matter. Therefore, in order to confirm this supposition, we calculated the ratio between the

percentage of the decomposed organic carbon and the percentage of the ammonified nitrogen, as shown in Table IX.

TABLE IX.

Soy-bean cake in loam.

	Percentage of decomposed organic carbon after 4 weeks.	Percentage of ammonified N.	Ratio.	Average.
No lime.	53.36	51.36	1.03:1	1:1
CaO 1.0 %	70.40	77.02	0.92:1	
CaO 0.5 %	60.98	59.88	1.02:1	1:1
CaO 0.1 %	52.92	52.28	1.01:1	
$CaCO_3$ 1.0 %	67.26	92.87	0.74:1	
$CaCO_3$ 0.5 %	58.30	69.53	0.82:1	1:1
$CaCO_3$ 0.1 %	52.46	62.44	0.84:1	

Herring cake in loam.

	Percentage of decomposed organic carbon after 4 weeks.	Percentage of ammonified N.	Ratio.	Average.
No lime.	50.00	31.63	1.39:1	1.5:1
CaO 1.0 %	63.77	66.37	0.82:1	
CaO 0.5 %	58.16	40.45	1.44:1	1.5:1
CaO 0.1 %	54.08	35.31	1.54:1	
$CaCO_3$ 1.0 %	59.70	61.40	0.98:1	
$CaCO_3$ 0.5 %	56.12	42.75	1.32:1	1.2:1
$CaCO_3$ 0.1 %	51.53	38.39	1.56:1	

Soy-bean cake in acid soil.

	Percentage of decomposed organic carbon after 4 weeks.	Percentage of ammonified N.	Ratio.	Average.
No lime.	40.36	24.22	1.67:1	1.7:1
CaO 1.0 %	68.61	35.45	1.73:1	
CaO 0.5 %	56.50	30.62	1.85:1	1.8:1
CaO 0.1 %	51.57	29.11	1.77:1	
$CaCO_3$ 1.0 %	61.88	41.34	1.51:1	
$CaCO_3$ 0.5 %	56.95	33.79	1.68:1	1.5:1
$CaCO_3$ 0.1 %	43.93	30.44	1.44:1	

Herring cake in acid soil.

	Percentage of decomposed organic carbon after 4 weeks.	Percentage of ammonified N.	Ratio.	Average.
No lime.	49.49	16.24	3.05:1	3:1
CaO 1.0 %	82.65	27.79	2.98:1	
CaO 0.5 %	67.34	21.36	3.16:1	2.9:1
CaO 0.1 %	52.04	20.67	2.52:1	
$CaCO_3$ 1.0 %	80.10	28.85	2.79:1	
$CaCO_3$ 0.5 %	66.33	22.85	2.91:1	2.5:1
$CaCO_3$ 0.1 %	50.51	21.78	2.33:1	

According to these results, a constant ratio between the percentage of the decomposed organic carbon and the percentage of the ammonified nitrogen is not observable. But when, both for loam and acid soil, soy-bean cake and herring cake, the relative number of the percentage of the decomposed

organic carbon, calculated by assuming that the percentage of the ammonified nitrogen was 1, was compared, a constant ratio, as shown in the following table, was found:—

In the case of soy-bean cake.

	In loam: In acid soil
In the case of no lime	1: 1.7≒1: 2
When CaO was added	1: 1.8≒1: 2
When $CaCO_3$ was added	0.8: 1.5=1: 1.9

In the case of herring cake.

	In loam: In acid soil
In the case of no lime	1.5: 3.0=1: 2
When CaO was added	1.5: 2.9=1: 1.9
When $CaCO_3$ was added	1.2: 2.5=1: 2

In the case of loam.

	Soy-bean: herring
In the case of no lime	1.0: 1.5
When CaO was added	1.0: 1.5
When $CaCO_3$ was added	0.8: 1.2=1: 1.5

In the case of acid soil.

	Soy-bean cake: Herring cake
In the case of no lime	1.7: 3.0=1: 1.7
When CaO was added	1.8: 2.9=1: 1.6
When $CaCO_3$ was added	1.5: 2.5=1: 1.6

Assuming that the percentage of ammonified nitrogen was 1, then the ratio between the percentage of the decomposed organic carbon in loam and in acid soil, was about 1:2 in all cases. Therefore, it might be said that:

1) Twice the amount of carbon of organic matter, which was decomposed to carbonic acid in loam, had to be decomposed in acid soil, to ammonify the same amount of nitrogen of the same organic matter.

If, on the other hand, the ratio between the percentage of decomposed organic carbon of soy-bean cake and of herring cake was almost 1:1.5 in all cases, then it might be said that:—

2) To ammonify the same quantity of nitrogen of organic matter, 1.5 times the amount of organic carbon of soy-bean cake which was decomposed in loam or acid soil had to be decomposed to carbonic acid in the case of herring cake.

In the same way, the percentage of the ammonified nitrogen which was calculated by assuming that the percentage of the decomposed organic carbon was 1, served as a means of comparison between the results of loam and acid soil, and, on the other hand, between the results of soy-bean cake and herring cake. The former ratio was about 2:1 and the latter 1.5:1. Therefore, we arrived at the following conclusions:—

1) In order to decompose the same amount of carbon of organic matter to carbonic acid, twice the amount of nitrogen which is ammonified in acid soil has to be ammonified in loam.

2) If the same amount of carbon of organic matter has to be decomposed, 1.5 times the amount of nitrogen of herring cake have to be ammonified in the case of soy-bean cake, irrespective of the kind of soil.

These four suggestions were derived from the experiments with soy-bean cake or herring cake in loam or in acid soil, and the authors thought that these findings might also apply to any kind of organic matter in soil. However, further investigations upon this subject are necessary.

Deductions.

1) Calcium oxide and calcium carbonate stimulate the decomposition of organic matter in soil.

2) Calcium oxide and calcium carbonate stimulate the ammonification and the nitrification of organic matter.

3) The effect of lime upon the decomposition of the carbon compound of organic matter or upon the ammonification of organic matter differed according to the kinds of soil (loam and acid soil), but not according to the character of the organic matter (soy-bean cake and herring cake); and

the effect of lime upon the nitrification was the same for the both kinds of soil, but differed according to the origin of the organic matter.

4. The effect of lime varied according to its combination form. CaO acted favorably upon the formation of carbonate which remained in the soil, while $CaCO_3$ stimulated the production of CO_2 gas. The effect of CaO upon the decomposition of the carbon compound was greater than that of $CaCO_3$, whereas the effect of CaO upon the ammonification and the nitrification was smaller than that of $CaCO_3$.

5. To ammonify the same amount of nitrogen of organic matter, twice the amount of organic carbon of soy-bean cake or of herring cake which was decomposed in loam had to be decomposed in acid soil to carbonic acid. On the other hand, to ammonify the same amount of nitrogen of organic matter, 1.5 times the amount of organic carbon of soy-bean cake which was decomposed to carbonic acid in loam or in acid soil had to be decomposed in the case of herring cake in the same soil. And this constant was calculated in the following way:—

Assuming that the percentage of ammonified nitrogen was 1, the ratio between the percentage of decomposed organic carbon and the percentage of ammonified nitrogen was calculated. Then these two relative numbers were put in relation and the constant determined.

6. With regard to the ammonification, we observed that the same relations existed by assuming that the percentage of decomposed organic carbon was 1.

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ON THE PREPARATION OF TESTES NUCLEIC ACID.

By

TETSUTARO TADOKORO and TEIZO TAKAHASHI.

(From the Institute of Agricultural Chemistry of Hokkaido Imperial University, Sapporo.)

(Received for publication, May 4, 1923.)

Animal nucleic acid which is widely distributed in almost all organs of the animal body has been isolated by many investigators such as A. Kossel and A. Neumann (1894), A. Neumann (1899), O. Schmiedeberg (1900), S. Kostytschew (1903), I. Bang (1904) and W. Jones (1908) from thymus gland; I. Bang (1898), O. V. Fürth and E. Jelsalem (1907), R. Feulgen (1913) and P. A. Levene (1901–1903–1921–1922) from pancreas; P. A. Levene (1901–1905–1921–1922) from spleen; M. Schmiedeberg (1896), K. Inoue (1906), P. A. Levene and J. A. Mandel (1905) from fish sperm; J. A. Mandel and P. A. Levene (1906) and P. A. Levene (1922) from kidney; P. A. Levene and J. A. Mandel (1905) from milk gland; P. A. Levene (1903-1921–1922) from liver; K. Inoue and Y. Kotake (1905) from intestinal slime membrane; R. Nakaseko (1918) from lymphatic corpuscle; T. Kikkoji (1907) from human placenta; P. A. Levene (1903) from brain; P. A. Levene (1903) from testes; O. Schmiedeberg (1908) from spleen, thymus, pancreas and thyroid gland of salmon; W. G. Ruppel (1898–99), P. B. Johnes and E. B. Brown (1922) from tubercle bacilli. Besides these reports many investigators have isolated animal nucleic acids for the purpose of further study of chemical, physical and physiological properties. But here we have no space to describe the details of these results.

But the main sources employed for the preparation of animal

nucleic acid are thymus gland, pancreas and spleen. The preparation of a pure animal nucleic acid remained a difficult task notwithstanding that many methods have been recommended, in recent years. Among these sources above mentioned, testes of higher animals which are apparently thought to be an adequate material for the preparation of nucleic acid, have been neglected in use. This is probably on account of the difficulty of preparation in pure state and consequently small amount in yield. The only investigator who has undertaken the study of the nucleic acid of testes was P. A. Levene (1903) (1904), he has not only isolated testes nucleic acid from testes of ox, but also subjected same to hydrolysis and succeeded in isolating purine and pyrimidine bases and laevulic acid. But he never determined the other elementary constituents except phosphorus of the material obtained. He describes in his report as follows:—"Die Hauptaufgabe war zunächst, eine für die hydrolytische Spaltungsprodukte zu finden sind. Es musste dabei die Bemühungen, ein ganz einheitliches und reines Präparat zu erhalten, in den Hintergrund treten." The details of the process and the results as carried out by him are briefly described as follows:—Fresh testes of ox which were dissected and made free from pellicle and ground, were subjected to the ordinary process of preparation of animal nucleic acid described by P. A. Levene (1902). That is, the ground material was boiled with 5 per cent sodium chloride solution for 1 hour and to this solution sodium acetate was added to make up to 10 per cent, then the solution was made a 5 per cent solution of sodium hydroxide and allowed to stand over night. The protein contained in the solution was precipitated with acetic acid and picric acid and then filtered. From this filtrate nucleic acid was precipitated with a solution of copper chloride. This crude nucleic acid was dissolved in alkaline solution and reprecipitated with 10 % hydrochloric acid. After repeating this process several times, the precipitate was washed with alcohol and ether and dried in vacuo. This substance

contained 8.75 and 8.5 % of phosphorus. He further hydrolysed this substance and isolated guanine and adenine as purine base and thymine and cytosine as pyrimidine base, and afterwards he also obtained laevulic acid from this substance. Except for Levene the authors have never been acquainted with any one who has succeeded in isolating nucleic acid from testes of such higher animals as mammalia. So the authors made an effort to work out an easier and more reliable method for isolating pure nucleic acid from testes of higher animals, and to investigate its character.

Phosphorus contained in the products obtained by several methods was determined for the purpose of estimating the degree of purity. The material used for this investigation was the testes of pig (*Sus Scrofa domesticus*) for the reason that it is easily obtained in comparatively large amounts, moreover, it has never been used for the source of nucleic acid.

Experimental Part.

We carried out at first the process proposed by P. A. Levene as described above, washing the crude nucleic acid with 50.95 %, absolute alcohol and ether respectively, dissolved in the water containing a minimum amount of sodium hydroxide, and precipitated with 10 % hydrochloric acid and washed as above mentioned. Repeating this process five times the products were burnt to ash by the method of A. Vozarik (1912) and phosphorus was estimated by molybdic method. The following results were obtained:—

	Sample.	$Mg_2P_2O_7$	P
Preparation I.	0.2200 *gm.*	0.0306 *gm.*	3.87 %
Preparation II.	0.1462 „	0.0226 „	4.30 „

Next we tried a process which was carried out by the

author under the direction of P. A. Levene at the Rockefeller Institute, New York City, in the year 1920. In this process picric acid is used as the precipitant of protein substances and nucleic acid is precipitated by 20 % hydrochloric acid. The following products were obtained.

	Sample.	$Mg_2P_2O_7$	P
Preparation I.	0.1021 *gm.*	0.0181 *gm.*	4.93 %
Preparation II.	0.1130 „	0.0172 „	4.24 „
Preparation III.	0.1215 „	0.0188 „	4.31 „
Preparation IV.	0.1000 „	0.0174 „	4.84 „
Preparation V.	0.1017 „	0.0149 „	4.08 „

Beside these we obtained several products which had about the same percentage of phosphorus. The yield obtained by this process was 2.5–2 gm. per 1 kg. of crushed testes. The products were then submitted to the process of purification as above mentioned, and the phosphorus content was determined.

	Sample.	$Mg_2P_2O_7$	P
Crude nucleic acid	0.1775 *gm.*	0.0275 *gm.*	4.31 %
Purified 3 times	0.1061 „	0.0188 „	4.85 „
„ „	0.1090 „	0.0162 „	4.80 „
Purified 4 times	0.0874 „	0.0162 „	5.16 „
Purified 6 times	0.0375 „	0.0086 „	6.38 „
Purified 9 times	0.0547 „	0.0115 „	5.85 „

In the course of this process the yield decreased considerably and from 1/3 to 2/3 of the original weight was lost in every purification process. Moreover we had another product purified 10 times which contained only 4.8 % of phosphorus.

Then the authors tried another method, considering that the low content of phosphorus depends upon the mixture of nuclein in the product, and that the boiling of the material with alkaline solution will be profitable, for nuclein is split by boiling with alkaline solution to protein and nucleic acid. For the purpose of preparing thymus nucleic acid A. Neumann (1899) boiled the sample with 1.7 % of sodium hydroxide for 30 minutes. So the authors tried the following process. 1 kg. of the fresh testes was kept at 95° C. with 3400 cc. of 1.7 % sodium hydroxide solution for half an hour. After cooling, nucleic acid was precipitated in the way already described and purified twice, but it reached no larger % of phosphorus than 4.23 and 3.6 %. Continuing the boiling for 2 hours, the product contained only 3.39 % of phosphorus. This product was dissolved in 2.5 % sodium hydoxide solution and neutralised with conc. acetic acid and the precipitate then formed was removed. The purification was repeated 6 times, but the product contained only 4.85 % of phosphorus. Recently P. A. Levene (1922), after testing several methods, proposed a modified method of preparing animal nucleic acid, and using this method he easily isolated nucleic acid from several kinds of organs as thymus gland, spleen, kidney, pancreas and liver. In this process colloidal iron solution (iron dialysed, 5 % $Fe_2 0_3$) is used and amylalcohol is substituted for ethyl alcohol. The authors, too, accepted this new process for the preparation of testes nucleic acid, but obtained unexpected results, owing partially to the unskilful technique. This method gave the following results.

	Sample.	Ash.	$Mg_2P_2O_7$	P
Preparation I.	0.1093 *gm.*	19.21 %	0.0128 *gm.*	3.26 %
Preparation II.	0.1131 „	19.13 „	0.0135 „	3.32 „

The crude nucleic acid was purified according to his method, and analysed.

	Sample.	Ash.	$Mg_2P_2O_7$	P
Preparation I.	0.0852 *gm.*	13.62 %	0.0100 *gm.*	3.27 %
Preparation II.	0.1030 ,,	18.06 ,,	0.0140 ,,	3.78 ,,

Considering the above results, the low content of phosphorus in the products made by several methods already described, depends on the mixture of protein which escaped from being precipitated by acetic acid and picric acid or colloidal iron solution, but which was precipitated by hydrochloric acid in comparison with nucleic acid. This protein, the authors thought, might be separated out well when boiled after acidifying fairly with acetic acid and picric acid. W. Jones (1912) boiled the material after acidifying with acetic acid when preparing thymus nucleic acid. Under these considerations, the alkaline extract solution of nucleic acid was neutralized with conc. acetic acid and more was added to make it distinctly acid to litmus paper. Then about 300 cc. of hot saturated picric acid solution were added. After boiling this solution it was filtered rapidly and the filtrate was diluted to double volume and the nucleic acid was precipitated by the ordinary method. The products had the following content of phosphorus:—

	Sample.	$Mg_2P_2O_7$	P
Preparation I.	0.1742 *gm.*	0.0324 *gm.*	5.18 %
Preparation II.	0.1686 ,,	0.0330 ,,	5.45 ,,
Preparation III.	0.1676 ,,	0.0376 ,,	6.25 ,,
Preparation IV.	0.0800 ,,	0.0180 ,,	6.26 ,,

These products were submitted to the ordinary process of purification 3 times and gave the following results.

	Sample.	$Mg_2P_2O_7$	P
Preparation I.	0.1340 *gm.*	0.0350 *gm.*	7.27 %
Preparation II.	0.1735 „	0.0459 „	7.36 „
Preparation III.	0.0965 „	0.0268 „	7.73 „

There is fear of decomposing nucleic acid when boiling with an addition of acetic acid resulting in decreased yield. Acetic acid is replacable by picric acid for the precipitation of protein. The authors modified, as follows, the above described method. 1 kg. of the crushed sample was boiled with 3400 cc. of 5 % sodium chloride solution. 40 % sodium hydroxide solution was added to make a 5 % solution, and this was allowed to stand over night. Next morning the solution was neutralized with conc. acetic acid and acidified fairly and heated up to 85° C., then instantly filtered. From the filtrate nucleic acid was precipitated with 20 % hydrochloric acid as in the ordinary way. It was immersed in 50 % alcohol, 95 % alcohol and a mixture of alcohol and ether for half a day respectively, and then was washed with absolute alcohol and ether and dried in vacuo. The products of this method were as follows:—

	Sample.	Ash.	$Mg_2P_2O_7$	P
Preparation I.	0.1048 *gm.*	21.19 %	0.0250 *gm.*	6.64 %
Preparation II.	0.1010 „	19.18 „	0.0234 „	6.54 „

It may be said that the above method is profitable for the preparation of testes nucleic acid. The crude nucleic acid which was obtained by this method was dissolved in water (1 gm. in 150 cc.) adding a minimum amount of sodium hydroxide and acidified with conc. acetic acid and heated up to

85° C. and instantly filtered. The coagulated substance separated out by this time gave a very marked biuret reaction. From the filtrate nucleic acid was precipitated in the usual way. This process of precipitation requires a considerable tract, but is very convenient for abbreviating the repeating process of purification. The yield of crude nucleic acid about 2 gm. per 1 kg. of fresh testes, and pure nucleic acid was about 0.4 gm. In the following tables are given the results of the analyses of the samples obtained by this procedure.

Analytical Part.

Phosphorus was estimated by molybdic method after burning to ash, nitrogen was estimated by Kjeldahl's method.

	Sample.	Ash.	$Mg_2P_2O_7$	P	Sample.	N	N
Preparation I.	0.0856 *gm*	21.34 %	0.0570 *gm*	8.55 %	0.0500 *gm*	0.007314 *gm*	15.03 %
Preparation II.	0.1878 ,,	22.11 ,,	0.0274 ,,	8.69 ,,	0.0500 ,,	0.007514 ,,	15.03 ,,

	Sample.	CO_2	H_2O	O	H
Preparation I.	0.1160 *gm.*	0.1614 *gm.*	0.0516 *gm.*	37.95 %	4.94 %
Preparation II.	0.0990 ,,	0.1386 ,,	0.0407 ,,	38.18 ,,	4.57 ,,

The nucleic acid obtained by the method above described has the following properties;

1) Hardy soluble in water, insoluble in alcohol and ether but easily soluble in NaOH, KOH, NH_4OH and sodium acetate solutions.

2) The coagulated precipitate by hydrochloric acid from alkaline solution is very viscous and has a silky lustre, and becomes a brownish or reddish white powder after drying in vacuo. It is heavier than protein.

3) Biuret and Millon's reactions are negative.

4) Never precipitated by acetic acid but precipitated by HCl, H_2SO_4 and HNO_3 from its alkaline solution.

5) Treating with hydrochloric acid and pholoroglucin, it has a pentose-like very slight colour reaction.

6) It reduced no Fehling's solution after treating with mineral acids for a short time.

Distribution of nitrogen of the testes nucleic acid which was determined by the method proposed by Hausmann as modified by Osborn and Harris (1903), will be compared with the nucleic acids of other sources, as follows:—

	Total-N.	Total Nitrogen as 100.						
		20 % HCl soluble-N.	insoluble-N.	Ammonia-N.	Humin-N.	Basic-N.	non-basic-N.	P
Testes nucleic acid	15.03 %	100.00 %	0 %	4.00 %	8.68 %	48.08 %	39.24 %	8.55%
Thymus nucleic acid.....	15.93 ,,	98.49 ,,	1.51 ,,	7.28 ,,	10.77 ,,	48.27 ,,	32.77 ,,	8.35 ,,
Yeast nucleic acid	16.57 ,,	97.89 ,,	2.11 ,,	10.44 ,,	7.66 ,,	55.16 ,,	24.63 ,,	8.17 ,,

Finally the authors applied this process for the preparation of nucleic acid from other origins, liver and yeast. Liver is said to be an unfavorable material for the production of pure nucleic acid on account of the large proportion of carbohydrate and other substances. In the following tables are given the results of determination of phosphorus and nitrogen.

	Sample.	Ash.	$Mg_2P_2O_7$	P
Preparation I.	0.1278 *gm.*	15.65 %	0.0304 *gm.*	6.62 %
Preparation II.	0.1270 ,,	15.43 ,,	0.0271 ,,	5.94 ,,

After the treatment of the purification process the products gave the following results.

Sample.	Ash.	$Mg_2P_2O_7$	P	N
0.0623 *gm.*	19.42 %	0.0200 *gm.*	8.94 %	—
0.0500 „	—	—	—	15.04 %

Yeast nucleic acid was made by the following process. 0.5 kg. of fresh yeast was well washed with water, immersed in 2 litres of 2.5 % sodium hydroxide solution and shaken 10 minutes. It was neutralyzed with conc. acetic acid and acidified faintly with acetic acid and heated up to 85° C. and instantly filtered. To the filtrate magnesium sulphate (40 gm. per 1 litre) was added, the nucleic acid was precipitated with 20 % hydrochloric acid and treated in the usual way and analyzed.

	Sample.	Ash.	$Mg_2P_2O_7$	P
Preparation I.	0.1278 *cm.*	15.65 %	0.0304 *gm.*	6.62 %
Preparation II.	0.1024 „	23.92 „	0.0280 „	7.61 „

The crude nucleic acid was dissolved in water adding a minimum amount of alkalin and acidified with conc. acetic acid; in this case the heating may be abridged. From the filtrate nucleic acid was precipitated with 20 % hydrochloric acid and washed and dried and we obtained the following results.

Sample.	Ash.	$Mg_2P_2O_7$	P	N
0.0500 *gm.*	20.16 %	0.0145 *gm.*	8.07 %	—
0.0500	—	—	—	15.04 %

Summary.

1. Several methods were tried for the preparation of

nucleic acid from testes of pig, in pure state, and it was found that heating the extract solution with an addition of the proper amount of acetic acid is more effective and easier for the removal of protein substance. The same method was also easily applicable for the preparation of nucleic acid from other sources, liver and yeast. The process of purification was also modified.

2. Nucleic acid was isolated from testes of pig, and it was submitted to qualitative analysis. The elementary composition and distribution of nitrogen were determined.

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SOME ANALYTICAL STUDIES ON THE URINARY CALCULUS.

By

MASAO IWANO.

(*From the Biochemical Laboratory, Institute of Medical Chemistry, Tokyo Imperial University. Director: Prof. S. Kakiuchi.*)

(Received for publication, May 17, 1923.)

I. Introduction.

At the request of Assistant-professor H. Nakano of the Urological Institute of this University the writer was engaged during the Summer of 1921 in the chemical analysis of the components of the urinary calculi. The total number of calculi taken for the analysis amounted to 487 and they were gathered from widely separate districts of Japan. In this paper the results of this analysis are reported and theoretical considerations on some factors with respect to the formation of the calculus are offered.

Since it seemed to the writer unreasonable to draw any conclusion from the analytical data obtained on a calculus as a whole, though frequently met with in the old literatures in this line, the writer undertook the analysis on each layer appearing in the cutting face through its longitudinal axis of the calculus.

In the simple or mixed up calculus, which indicates no laminentation, the central and peripheral parts were separately analysed.

II. The Qualitative Analysis of Calculi.

From each layer of a calculus a small portion was scraped out and examined as follows.

Uric Acid and *Urate.* A small portion of the calculi was placed in a porcelain crucible, moistened with a few drops of concentrated nitric acid, evaporated to dryness and then tested for murexid reaction.

Phosphate was detected by production of a yellow, crystalline precipitate of ammonium phospho-molybdate. It is best to proceed as follows: To a minute quantity of the powder placed on a slide, the ammonium molybdate reagent is added, after warming, the crystal thus produced is examined microscopically.

Ammonium was detected by Nessler's reagent.

Carbonate was detected through the decomposition with effervescence by means of dilute hydrochloric acid. The reaction took place ordinarily in the cold and sometimes on warming.

Potassium and *sodium* were detected by their flame reactions.

Calcium was detected as follows: A lump of calculus was dissolved by means of dilute hydrochloric acid, a few drops of ammonium oxalate solution were added, made alkaline with ammonia and then acidified slightly with acetic acid. If calcium is present, a white precipitate of calcium oxalate will appear on standing or on warming.

Oxalate was detected as calcium oxalate as in the case of calcium, with difference of applying $CaCl_2$ instead of ammonium oxalate.

Magnesium was detected by producing a typical white crystalline precipitate of ammonium magnesium phosphate in the solution containing ammonium chloride, phosphate and ammonia.

Cystine was identified microscopically or by means of the lead sulphide reaction.

Some of these results are inserted in the following table.

TABLE I.

Samples No.	Age & sex	Macroscopic layer	Uric acid or urate	Phosphate	Oxalate	Carbonate	Cysenit	Na	K	NH_4	Ca	Mg
245	24 ♂	1	+++	+	−	−	−	+	+	+	−	−
		2	+++	+	−	−	−	+	+	+	±	−
		3	+++	+	−	−	−	+	+	+	−	−
304	56 ♂	1	+++	−	−	−	−	+	+	+	−	−
		2	+++	±	−	−	−	+	+	+	−	−
359	66 ♂	1	+	+++	−	++	−	++	++	+	+	+
		2	+	+++	−	++	−	++	++	+	+	+
		3	+	+++	−	++	−	+	+	++	+	+
		4	+	+++	−	++	−	+	+	++	+	+
246	37 ♀	1	−	+++	−	++	−	+	+	+	+	++
		2	−	+++	−	++	−	+	+	+	+	++
		3	−	+++	−	++	−	+	+	+	+	++
		4	−	+++	−	++	−	+	+	+	+	++
241	61 ♂	1	−	+	+++	±	−	+	+	±	+	−
		2	−	+	+++	±	−	+	+	±	++	−
		3	−	+	+++	±	−	+	+	±	+	−
284	10 ♂	1	+	+	+++	±	−	+	+	++	+	−
		2	+	+	+++	±	−	+	+	++	+	−
		3	+	+	+++	±	−	+	+	++	+	−
26	2 ♂	non	−	±	−	−	+++	−	−	+	−	−
502	25 ♂	,,	−	+	−	−	+++	+	+	+	+	−
22	6 ♂	1	++	++	−	−	−	+	+	+	+	−
		2	++	++	−	−	−	+	+	+	+	−
		3	++	++	−	−	−	+	+	+	+	−
		4	++	++	−	−	−	+	+	+	+	−
129	unknown	1	++	+	++	±	−	+	+	+	+	−
		2	++	+	++	±	−	+	+	+	+	−
		3	++	+	++	±	−	+	+	+	++	−
		4	++	+	++	±	−	+	+	+	+	−

Samples	Age & sex	Macroscopic layer	Uric acid or urate	Phosphate	Oxalate	Carbonate	Cystine	Na	K	NH_4	Ca	Mg
505	unknown	1	+++	+	−	−	−	+	+	±	±	−
		2	+	+	+++	−	−	+	+	+	++	−
		3	+	+++	−	−	−	+	+	+	++	−
		4	+	++	++	−	−	+	+	+	++	−
		5	+	++	++	−	−	+	+	+	++	−
119	36 ♂	1	−	+	−	−	+++	−	+	+	+	−
		2	−	++	−	±	++	−	+	+	+	−
		3	−	+++	−	−	−	+	+	+	++	−
169	43 ♂	1		Foreign body (Cottonstring)								
		2	−	+++	−	+	−	++	++	+	++	+
		3	−	+++	−	+	−	++	++	+	++	+
498	40 ♀	1		Foreign body (hairs)								
		2	−	+++	−	+	−	+	−	+	++	+

It will be seem from this table that the the urinary calculus was chiefly composed of urate, phosphate, Oxalate and carbonate of alkaline earth metals such as Na, K, NH_4, Ca and Mg and of cystine, though the latter was rarely found.

In most cases, especially in phosphate and cystine calculi, the organic frame work was always present and when the adequate mass of calculi was dissolved away in dilute mineral acid or alkali, it came out as loose flocculi. The uric acid, urate and oxalate concretions were always contaminated with certain pigments.

In a few cases, some foreign bodies such as straw, paper-strings, cotton-string, wax and gauze were observed in a centre of the concretions, apparently serving as a nucleus in the formation of the calculus. The peripheral part of such calculus mostly consisted of phosphate.

As to the mode of mixture of each constituent composing the same calculus, the following points are to be noticed.

The *phosphate* existed in nearly all calculi except one, which was formed simply of uric acid or urate. The *uric acid* or *urate* was present alone or when mixed, chiefly with oxalate or phosphate, but never with cystine and carbonate. The *carbonate* occurred in most cases as calcium salt associated with the phosphate and was, striking enough to mention, always with its magnesium salt. The *cystine* found itself free or in company with various amounts of phosphate and when the latter attained to a considerable amount, a trace of carbonate was sometimes present. None of xanthine-, indigo-, carbonate- or cholesterol calculus, though they have been occasionally given in the literatures, was present in our cases.

It can be therefore concluded from the above statements, that there exists a definite relation as to the mixture of each constituent. The following table is brought forward for clear understanding of this relation.

TABLE II.

Sort of Simple calculi.	Components									
	Urate.	Phosphate.	Oxalate.	Carbonate	Cystine.	Na	K	NH_4	Ca	Mg
U, Urate.	+	+	+	−	−	+	+	+	+	−
Phosphate.	+	+	+	+	+	+	+	+	+	+
Oxalate.	+	+	+	−	−	+	+	+	+	−
Cystine.	−	+	−	−	+	+	+	+	+	−

III. The Colour and glossy Appearance of the Urinary Calculus and their Relation to the Chemical Composition.

The calculus containing uric acid or urate is stained yellow, yellowish brown or brownish red; that of oxalate, dark brown or brownish black; that of phosphate, white or

grayish white, when in the form of magnesium salt, violet or pale green and of marble appearance; that of cystine, yellow or brownish yellow and of wax-like gloss. The experience has led the writer easily to distinguish the chemical constitution of each portion of the calculus at a glance in perfect agreement with the analytical data.

IV. The Quantitative Analysis of Calculi.

On 15 typical concretions the quantitative analyses were undertaken. The methods applied for each constituent are described briefly as follows.

1) *Water content.* An aliquot of calculus was placed in a porcelain-crucible and weighed, then dried at 100–110°C to constant weight. Substracting this value from the former, the water content of calculus was obtained.
2) *Phosphate* was determined both by the method of the Neumann's alkalimetry (1902) and the Classen's acidimetry. (1912)
3) *Carbonate* was determined by the Fresenius-Classen's method.
4) *Uric acid* and *urate*, by the Folin-Macallum's microchemical colorimetric method. (1913)
5) *Oxalate*, by the Salkowski-Autenrieth-Barth's method.
6) *Cystine*, by the Gaskel's method. (1907)
7) *Sodium* and *potassium*, by the combination of the methods of Lehmann (1884) and Green. (1912)
8) *Calcium* and *Magnesium*, by the McCrudden's method. (1911)
9) *Ammonium*, by the micro-chemical method of Folin-Macallum. (1912)
10) *Total nitrogen content*, by the colorimetric micromethod of Folin-Farmer. (1912)

The results are summed up in the following table.

TABLE III.

Sorts of calculi.	Samples. No.	Percentage of chief components.											
		Uric acid.	P_2O_5.	Oxalic acid.	Carbonic acid.	Cystine.	Na	K	NH_4	Ca	Mg	H_2O	Total N.
Urate	35	85.12	0.43	—	—	—	1.27	0.17	0.04	0.25	—	1.08	29.37
	78	73.67	0.40	—	—	—	0.74	0.10	—	0.57	—		26.81
Phosphate	135	—	32.60	—	7.91	—	3.61	0.42	0.90	20.63	0.46	13.71	2.51
	386	2.50	30.71	—	3.53	—	4.50	0.30	0.76	15.80	5.26	20.12	2.74
	387	2.53	29.21	—	3.88	—	5.31	0.14	1.16	7.36	6.29		3.68
Mixed	44	18.37	21.33	—	1.16	—	14.16	0.27	—	7.21	—		8.32
	315	14.15	16.31	17.55	2.01	—	2.58	0.11	0.89	22.68	0.18		6.38
Oxalate	36	10.22	3.53	35.08	—	—	3.28	0.32	—	19.47	—	5.91	6.32
	29	3.50	1.87	40.53	—	—	1.21	0.15	—	20.31	—		3.28
C stine	94	—	1.30	—	—	80.10	2.80	0.10	—	1.02	—	2.26	10.73
	119	—	6.12	—	—	62.70	7.55	0.11	0.21	5.84	—		9.36

The first column of the above table indicates the kind of calculus judged from its colour and glossy appearance. The results of the quantitative analysis show quite a coincidence.

From Table III it is known that the concretion, which was assumed to be an uric acid or urate calculus by its appearance, consists in greater extent of uric acid itself, and when combined, though in slight degree, of Na, K and NH_4.

The *phosphoric acid* existed partly as secondary and mostly as tertiary phosphate of the alkali metals (Na, K, NH_4) and the alkaline earth metals (Ca, Mg).

The *oxalic* and *carbonic acid* was present in combination with calcium, while the cystine existed free or in combination with the alkali metals.

It is also remarkable that there are some discrepancies between the value of nitrogen found by the Kjehldahl method and the total sum of nitrogen derived from uric acid, urate, NH_4 or cystine. This is demonstrated in the following table.

TABLE IV.

Samples No.	Percentage of nitrogen in					
	Uric acid	NH_4	Cystine	Sum	Total	Difference
35	28.09	0.03	—	28.12	29.37	1.25
79	24.53	—	—	24.53	26.81	2.28
135	—	0.70	—	0.70	2.51	1.81
386	0.83	0.59	—	1.42	2.73	1.31
387	0.84	0.93	—	1.77	3.70	1.93
44	6.12	—	—	6.12	8.32	2.20
315	4.71	0.69	—	5.40	6.38	0.98
29	1.16	—	—	1.16	3.28	2.12
36	3.40	—	—	3.40	6.34	2.93
94	—	—	9.41	9.41	10.72	1.31
114	—	0.16	7.37	7.53	9.36	1.83

Probably this difference might be due to the presence of the urine pigments in the cases of uric acid, urate and oxalate calculi, while in the case of phosphate or cystine calculi, where no such pigmentation was observable, the nitrogen derived from so-called "frame-work" substance, the existence of which was assumed in the qualitative analysis, may be its cause.

V. The Classification of Calculi.

Urinary calculi are commonly classified according to their prominent component. But such a classification is not tenable for the laminated calculus, because each of the lamellae can indicate different compositions. Taking into consideration the structural and chemical properties of calculi, the following classification seems to suit better.

				Number of our samples
Calculi	I. Non-laminated calculi	a. Simple calculi	1. Uric acid (urate) calculi	88
			2. Phosphate calculi	78
			3. Oxalate calculi	80
			4. Cystine calculi	9
		b. Mixed calculi		26
	II. Laminated calculi			187
	III. Foreign body calculi			19

As a general rule of the above classification the following points are to be considered.

1) All calculi, except those containing foreign bodies, are classified into two main groups; *laminated* and *non-laminated*. Of course, there are also some calculi, which can not be determined at once. In such cases, the analytical results are to be consulted and if then there are apparent differences in chemical constituents in each portion, though a stratification is imperfect, such a calculus is called laminated.

2) The foreign body calculi are excluded from the above classification on account of their special formation.
3) The non-laminated calculi are subdivided into the *simple* and the *mixed*, the former including those calculi, which consist chiefly of a single constituent such as uric acid, urate, phosphate, oxalate and cystine, whereas the latter is built up from two and more constituents, which are all well mixed.
4) The laminated calculi are named by the constituents of each layer. For example, when a calculus is composed of three layers, which are urate, urate-oxalate, and phosphate in the order from centre to periphery, it is called urate-urano-oxalate-phosphate calculus.

VI. Some Considerations on the Formation of Urinary Calculi.

1. The chemical constituent of the nucleus and its relation to the age.

Since it seemed to the writer that the investigation of the constituents of the nucleus is of great importance with regard to its formation, much attention was paid to this subject. In the following table the relation between the the chemical constituents and the age is indicated.

TABLE V.

Age.	Number of samples. (The number of female is indicated in bracket)	Number of nuclei composed of					
		Uric acid and urate	Phosphate	Oxalate	Mixed	Cystine	Foreign body
1–10	51 (4)	12	0	19	18	2	0
11–20	38 (1)	7	2	19	10	0	0
21–30	48 (4)	7	10	22	7	1	1

Age.	Number of samples. (The number of female is indicated in bracket)	Number of nuclei composed of					
		Uric acid and urate	Phosphate	Oxalate	Mixed	Cystine	Foreign body
31-40	61 (10)	8	11	26	9	3	4
41-50	47 (4)	5	12	17	11	0	2
51-60	47 (4)	14	11	14	6	0	2
61-70	37 (6)	16	7	9	4	0	1
71-80	10 (2)	5	3	1	0	1	0
Sum	339 (35)	74	56	127	65	7	10
Samples at unknow age	148 (5)	35	33	47	22	2	9
Total sum	487 (40)	109	89	174	87	9	19
Percentage	8.2	22.4	18.3	35.7	17.9	1.8	3.9

Since the age of the person from whom each stone was taken indicates only when the operation was carried out, it has usually no direct bearing on the beginning age of the formation of the calculus. Hence all calculi are grouped together for each decennium. Now it appears from the table that uric acid and urate nuclei appear much more at advanced age, while phosphate nuclei occur most frequently in person of ages from 30 to 60 years and far less in young persons. Oxalate nuclei are formed most frequently in persons between the first and the fifth decennium. In the case of the mixed nuclei, the nature of components and the age of the patient reveal the following relation as shown in the next table.

TABLE VI.

Age.	Number and components of mixed nuclei.			
	Urato-oxalate	Oxalato-phosphate	Urato-phosphate	Urato-oxalato-phosphate
1-10	12	0	2	4
11-20	4	0	3	3
21-30	3	2	2	0
31-40	4	1	4	0
41-50	2	3	6	0
51-60	2	1	3	0
51-70	1	0	3	0
Sum	28	7	23	7

The number of nuclei composed of oxalate lies at the top of all and the number of nuclei made of other substances decreases in the order of uric acid (urate), mixed, phosphate, foreign body and cystine. According to the Ultzmann's report, nuclei composed of urate are found in 441 cases of the 545 calculi; those of phosphate, in 47; those of oxalate, in 31; those of foreign body, in 18; and those of cystine, only in 8.

On comparing Ultzmann's results with ours, no large difference is present in the number of foreign body and cystine nuclei, but the number of oxalate nuclei in our case is about 6 times greater than in his case; that of phosphate, 2 times greater. On the contrary, our samples of uric acid (urate) are equal to 1/3.5 of his. He did not distinguish between simple and mixed calculi. Even, therefore, if all the number of the mixed nuclei in our case is counted as that of uric acid (urate) nuclei, the number of these uric acid (urate) nuclei yet does not reach the half of his. This

prominent difference in the number of oxalate and uric acid (urate) nuclei might be ascribed to the different diet, on which European and Japanese live. Since the food of the former generally contains more meat, their urine is likely to be oversaturated by uric acid and the reaction of their urine tends to become acid, which favours the precipitation of uric acid and urate. On the contrary, the urine of vegetarians, and most Japanese are inclined to vegetarianism, is frequently overcharged with oxalate and the reaction tends to become alkaline, which leads to the precipitation of oxalate and phosphate, though the quantity of the latter in urine is much reduced in comparison with that of meat diet.

2. The composition of the central part of calculi formed in woman and its relation to the age.

The occurrence of urinary calculi in woman is rare owing to the anatomical relation of the urethra as already known. In our case the number of calculi formed in women is 8.2 per cent of the whole, being about 2–3 per cent larger than in the cases of Osterlen and others. And it is remarkable that a majority of the nuclei was especially composed of phosphate as shown in Table VII. As above demonstrated, since the formation of concretion and its growth in woman to a considerable size premises some special changes of urethra, bladder and urine, it might be well assumed that such conditions as cystitis gonorrhoica, pregnancy or paralysis of the bladder may play an important role in these connections. For such factors are in more or less degree correlated with the stagnation of urine and consequently apt to change its reaction into an alkaline one, they furnish to the urine most favourable conditions for the precipitation of phosphate, especially so in the case of Japanese, owing to the influence of vegetable diet on the urine. The above assumption is supported by the two facts that the urinary concretion in woman occurs most frequently at the ages, when

the women are mostly exposed to the danger of such affliction, and that the occurrence in our women is somewhat larger than in those of European countries.

TABLE VII.

Age.	Number of samples.	Components of nucleus and number of each component.					
		Uric acid.	Phosphate.	Oxalate.	Mixed.	Cystine.	Foreign body.
1-10	4	0	0	1	2	1	0
11-20	1	0	1*	0	0	0	0
21-30	4	0	2	1	1	0	0
31-40	10	0	7	0	2	0	1
41-50	4	0	3	0	1	0	0
51-60	4	2	2	1	0	0	1
61-70	6	0	2	1	0	0	1
71-80	2	0	2	0	0	0	0
Sum	35	2	19	4	6	1	3

* This sample was found in a woman of 20 years.

3. The relation between the constituents of central and peripheral part in the calculus.

Since each layer in the calculi represents the various stage of growth under different conditions, it is of interest to elucidate the relationship between the layers. In the following table, the main constituents in the nucleus and the superficial crust were brought together.

TABLE VIII.

Portion	Uric acid and urate	Phosphate	Oxalate	Carbonate	Cystine	Foreign body	Na	K	NH_4	Ca	Mg
Central	320	429	238	339	9	19	399	399	347	454	121
Peripheral	357	479	209	381	9	0	404	397	374	452	185

As clearly seen from this table, uric acid (urate), phosphate and carbonate are found in greater amount in the peripheral part than in the central, while with oxalate the reverse is the case.

As to the alkali and alkaline earth metals, they are all distributed equally through the calculus with the exception of ammonium and magnesium, which seem to predominate in the peripheral. This fact tells us that the nucleus once produced tends to cause inflammation on the surface of the urinary system, in which a nucleus is present, and the change of reaction and other colloidal behaviours of urine follow, thus leading to the deposition of phosphate and carbonate.

SUMMARY.

1. The analytical result of 487 urinary calculi is given.
2. A new classification of urinary calculi is advanced.
3. Oxalate nuclei are found in Japan most abundantly in contradiction to the current view.
4. The urinary calculi in women are almost all phosphate calculi. The explanation on this phenomenon is afforded.

The writer wishes to thank Professor S. Kakiuchi for advice throughout the course of the investigation.

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STUDIES ON PHLORHIZIN GLYCOSURIA.

I. Phlorhizin glycosuria and Renal activity.

By

TAKEYOSHI NAGAYAMA and TOSHITERU YOKOTA.

(*From the Laboratory of Biological Chemistry of the Tokyo Jikei-kwai Medical School.*)

(Received for publication, May 22, 1923.)

Introduction.

Since the publication of the memorable work of v. Mering (1886–1887) on the "Phlorhizin diabetes," various attempts have been made to find an accurate explanation for the action of phlorhizin. O. Minkowski (1893) stated that phlorhizin exerts no influence upon pancreas because in the pancreaticotomised animal the phlorhizin injection causes still further increase of sugar in urine. Lepine (1896) has given evidence that the sugar centre has no relation to the phlorhizin-glycosuria by reason of the fact that there is no change in the amount of sugar eliminated in urine when the cervical or thoracic cord is cut. J. E. Sweet and A. J. Ringer (1913) showed that the liver is not related to the phlorhizin-diabetes.

N. Zuntz (1895) pointed out that this glycosuria is due to the increased permeability of kidney for sugar. He injected phlorhizin into one renal artery and examined the urine from each kidney separately. The kidney on the injected side secreted sugar at once, while the secretion of sugar in the kidney on other side occurred after a short time. Levene (1894–1895) was of the same opinion with Zuntz, that phlorhizin can cause glycosuria by acting directly on the kidneys. But he suggested that this action of phlorhizin might find an explanation in the assumption that the kidney comes to produce sugar in itself. This assumption is shared by Zuntz, who stated in his text book that the secretion of glucose in

kidney is probably an active process and that this process is in some way stimulated by the phlorhizin.

On the other hand, Coolen (1895) found that blood sugar increased by the injection of phlorhizin even when the kidneys had been totally extirpated, and concluded that the phlorhizin-glycosuria might be probably due to the same factor which prevails in the case of general metabolic diabetes, so that the kidney is of minor significance.

Recently Nash (1922) performed a series of exact experiments proving that the content of sugar in the renal vein of phlorhizinised animals is less than in the blood of general circulation, and concluded that the phlorhizin glycosuria is certainly due to the increased permeability of kidney for sugar.

At all events the current idea on phlorhizin glycosuria tends to the acknowledgment of the renal theory.

Now, if this theory be correct, the question arises as to how the renal activity is modified in such a case. For example, does the excretion of urea undergo any change? With regard to this point Mathews (1916) states that the urea-secreting function of the kidney is increased by phlorhizin, while Goto and Kuno, on the basis of their experimental results on quite healthy adults, insist, that the renal function showed an alteration in the exceedingly low renal threshold only for sugar. Sakaguchi, Ito and Kuno's experiments with the patients of renal diabetes confirmed also that the functions of the kidney remained intact except that of eliminating sugar.

Since it seems worth while to examine this relation the authors performed the following experiments on rabbits.

Method of Experiment.

As experimental animal we used male rabbits of about 2 kg. weight. They were fed on Okara (a preparation from

bean) and about 15 hours before the experiment no food was given. The renal activity was examined by Addis, Burnet and Shevky's method (1918).

At the beginning of the experiment, 10–20 cc. of warm water solution or of an emulsion in olive oil of 0.25–0.5 gm. phlorhizin were subcutaneously injected into one group of rabbits, while as control the same amount of water or oil without phlorhizin was injected into the same group of rabbits. The sugar in urine was quantitatively estimated by Pavy-Kumagawa-Sudo-Momose's method or by Sudo's method.

The experimental results are recorded in detail in Table I, and summarised in Table II with accompanying Figure I.

TABLE I.

Effect of phlorhizin on the ratio: $\frac{\text{Urea in one hour's urine}}{\text{Urea in 100 cc. of blood}}$

1. Control experiments.

Rabbit.	Urine volume.	Urea in urine per hour.	Urea in blood. per 100 cc.	Ratio.	Sugar in urine.
		Period I.			
	cc.	*mg.*	*mg.*		*per cent*
1	6.0	147	—	—	0
2	31.0	549	180	3.05	0
3	10.8	220	291	0.76	0
4	14.6	307	216	1.42	0
5	3.5	163	216	0.316	0
6	26.0	663	300	2.21	0

Rabbit.	Urine volume.	Urea in urine per hour.	Urea in Blood per 100 cc.	Ratio.	Sugar in Urine.
			Period II.		
	cc.	*mg.*	*mg.*		*per cent*
1	12.0	320	222	1.44	0
2	14.0	583	195	2.99	0
3	14.6	416	336	1.24	0
4	20.0	630	291	2.16	0
5	10.2	184	291	0.635	0
6	18.5	755	297	2.54	0
			Period III.		
1	16.6	501	228	2.25	0
2	15.0	599	180	3.33	0
3	12.7	438	291	1.51	0
4	16.4	605	261	2.32	0
5	14.3	287	330	0.869	0
6	14.5	666	267	2.49	0
			Period IV.		
1	31.0	946	180	4.15	0
2	22.5	1040	177	5.88	0
3	25.8	1022	288	3.55	0
4	26.5	1057	228	4.64	0
5	42.0	794	345	2.30	0
6	23.0	1201	213	5.60	0

2. Experiments with Phlorhizin

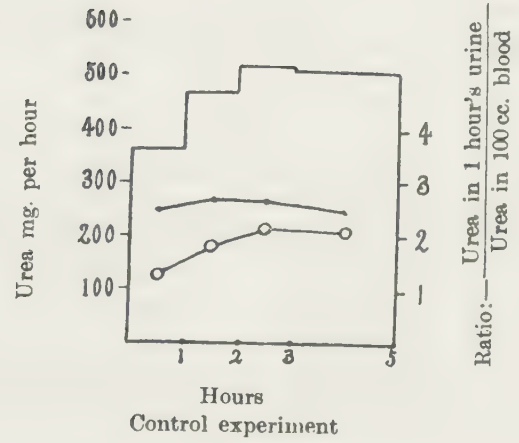
Rabbit.	Urine volume.	Urea in urine per hour.	Urea in Blood per 100 cc.	Ratio	Sugar in Urine
			Period I		
	cc.	*mg.*	*mg.*		*per cent*
1	13.0	324	186	1.79	1.65
2	38.0	456	219	2.08	1.09
3	16.2	272	264	1.03	1.14
4	18.6	363	228	1.59	2.04
5	5.3	111	300	0.37	0.67
6	32.0	634	297	2.13	1.38
			Period II		
1	12.0	378	210	1.80	0.52
2	22.0	568	222	2.55	1.56
3	19.6	435	288	1.51	1.07
4	17.4	475	246	1.83	1.83
5	9.4	200	315	0.634	0.79
6	24.0	734	294	2.49	1.92
			Period III		
1	12.6	510	240	2.13	0.39
2	19.6	700	207	3.82	1.67
3	16.0	374	288	1.30	1.25
4	13.8	493	261	2.00	2.14
5	14.1	258	342	0.754	0.76
6	23.0	745	261	2.85	1.81

Period.	Urine volume.	Urea in Urine per hour.	Urea in Blood per 100 cc.	Ratio.	Sugar in urine.
		Period IV			
	cc.	*mg.*	*mg.*		*per cent*
1	25.0	929	198	4.69	0.083
2	30.0	1323	204	6.48	1.00
3	35.8	1053	273	3.86	1.25
4	25.8	975	252	3.87	1.62
5	32.0	672	342	1.96	0.82
6	31.5	1285	213	6.03	2.66

Averages of separate periods

TABLE II.

Period.	Urine volume.	Urea in Urine per hour.	Urea in Blood per 100 cc.	Ratio.	Sugar in urine.
		Control experiments			
	cc.	*mg.*	*mg.*		*per cent*
1	15.3	342	241	1.55	0
2	14.9	481	272	1.83	0
3	14.9	516	260	2.13	0
4	28.5	505	239	2.11	0
average.	18.5	461	251	1.90	0
		Phlorhizin experiments			
1	20.5	360	246	1.28	1.33
2	17.4	465	263	1.80	1.28
3	16.5	513	267	2.14	1.34
4	30.0	508	247	2.06	1.24
average.	21.1	464	256	1.84	1.30



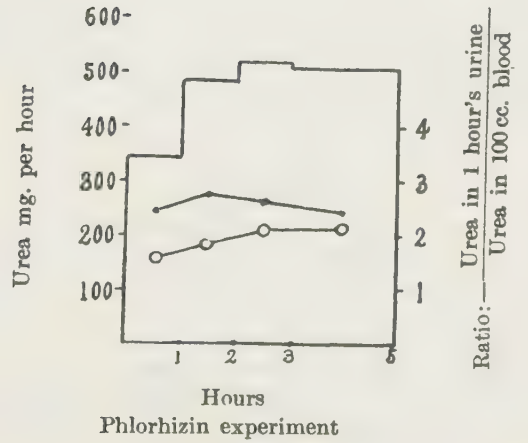


Fig. 1 shows the urea excreting activity of the kidney both in control and phlorhizin experiment. The ratio between the urea in one hour's urine and the urea in 100 cc. of blood is shown by circles joined by lines. The value is given on the scale at the right of the charts. The hourly rate of urea excretion is represented by the blocked areas. The blood urea concentration is shown by dots joined by lines. The value for both the hourly rate of urea excretion and the blood con-

centration are given by the scale on the ordinates at the left of the charts.

Summary.

We have examined the urea excreting activity of the kidney comparing the ratio $\left(\frac{\text{Urea in one hour's urine}}{\text{Urea in 100 cc of blood}}\right)$ in control and in phlorhizised rabbits. It showed, as we can see in Table I and II, that the ratio in the control gives an average 1.90, while that in the case of phlorhizinised rabbits gives 1.84. It is evident from the results that the phlorhizin has no appreciable effect on the urea excreting activity of the kidney.

The average amount of sugar eliminated in urine by phlorhizinised rabbits was 1.30 %.

A slight diuresis after phlorhizin injection, as previously well known, occurred also in these cases.

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STUDIES ON SOME FACTORS IN THE COAGULATION OF BLOOD.

By

KANAME KUWASHIMA.

(From the Biochemical Laboratory, Institute of Medical Chemistry, Tokyo Imperial University. Director: Prof. Samuro Kakiuchi.)

(Received for publication, December 13, 1922.)

Recently the question of coagulation of blood has been studied by many authors with some promising results. The identification of the thromboplastic substance (thrombokinase) with kephalin became quite probable by the work of Howell (1916), McLean (1916) and Gratia-Levene (1922). Howell and his coworkers further assume the presence of antithrombin and antiprothrombin in the blood plasma and insist that its prohibiting action may be annulled by the thromboplastic substance. Mills and his coworkers reject, however, Howell's view and advocate the direct participation in the formation of fibrin, reviving the Wooldridge's view of tissue fibrinogen (Mills, 1921; Mills and Guest, 1921).

The author of this paper has been trying for last two years to elucidate the relation concerning certain factors in the blood coagulation without attempting to give any theoretical interpretation to it at present. In the following some of results of these experiments are reported.

I. The Effect of Electrolytes on the Blood Coagulation.

It is quite well known to us that the blood which is made incoagulable by addition of oxalate becomes coagulable upon the addition of a soluble calcium salt and that the excess of the salt again inhibits the coagulation. According to Sab-

batini there are minimal and maximal concentrations of calcium chloride below and above which coagulation is inhibited. The upper limit is said to be a 0.162 molecular solution, the lower about one thousandth part of this concentration.

This action of calcium is specific and salts of barium or magnesium are entirely inactive. The specificy of calcium is shared however by strontium salt. In the following the result of some experiments regarding the effect of electrolytes on the blood coagulation is reported.

1. The specificy of calcium is shared by strontium.

To follow the effect of salt of various alkali earths on the re-activation of the oxalated plasma quantitatively 4 cc. of oxalated plasma obtained as described below was pipetted out into a clean test tube and 1 cc. of the solution of $CaCl_2$, $SrCl_2$, $BaCl_2$ and $MgCl_2$ of different concentration was added. The mixture was put into a thermostat and time of complete coagulation was noted.

Oxalate plasma. A clean bottle of 2 litre capacity containing 100 cc. of 10 per cent potassium oxalate solution was filled carefully with fresh horse blood and the contents were mixed thoroughly. After a lapse of 24 hours the supernatant clear plasma was transferred into another clean bottle and preserved in an ice chest. The result of experiment is given in the following table. (Table I).

The minimal and maximal concentration of calcium below and above which coagulation is inhibited coincide with that of Sabbatini. The action of strontium is quite observable, while barium and magnesium are entirely ineffective.

2. The effect of electrolytes on the blood coagulation.

The retarding action of various electrolytes on the velocity of blood-coagulation was then studied. For this purpose 2 cc. of the oxalate plasma was added with 1 cc. of salt-solution

TABLE I.

Concentration of solution.	Time of coagulation.			
	$CaCl_2$	$SrCl_2$	$BaCl_2$	$MgCl_2$
0.01 *mol*	non coagulable	non coagulable	non coagulable	non coagulable
0.02	,,	,,	,,	,,
0.03	,,	,,	,,	,,
0.04	9′	,,	,,	,,
0.05	7′	93′	,,	,,
0.06	7′	31′	,,	,,
0.07	8′	26′	,,	,,
0.08	9′	23′	,,	,,
0.09	11′	24′	,,	,,
0.10	12′	25′	,,	,,
0.11	13′	27′	,,	,,
0.12	19′	30′	,,	,,
0.13	22′	37′	,,	,,
0.14	29′	41′	,,	,,
0.15	41′	51′	,,	,,
0.16	72′	60′	,,	,,
0.17	110′	70′	,,	,,
0.18	180′	119′	,,	,,
0.19	240′	153′	,,	,,
0.20	on the next morning coagulated	370′	,,	,,
0.25	incomplete coagulation	incomplete coagulation	,,	,,
0.50	non coagulable	non coagulable	,,	,,

of different concentration, well mixed and 1 cc. of $CaCl_2$ solution, the concentration of which was previously found by trial to be the most suitable one for the quickest coagulation. The tubes were then put into the thermostat of 25° and the time of the onset of coagulation was noted. The test with a little larger and smaller amount of calcium chloride than the most suitable one was also simultaneously performed. The result is shown in the following tables.

TABLE II.

NaCl.

Concentration of salt added.	Time of coagulation.		
	$CaCl_2$ 0.035 mol.	$CaCl_2$ 0.05 mol.	$CaCl_2$ 0.125 mol.
mol.	*min.*	*min.*	*min.*
0	23	10	21
0.005	23	10	21
0.01	23	10	21
0.025	23	10	21
0.05	23	10	21
0.1	24	11	21
0.15	24	12	21
0.2	24	12	22
0.25	24	12	26
0.3	25	14	36
0.35	27	18	52
0.4	30	20	62
0.45	32	24	72
0.5	34	27	79
0.6	40	38	158
1.0	140	127	250

TABLE III.

Na_2SO_4.

Concentration of salt added.	Time of coagulation.		
	$CaCl_2$ 0.035 mol.	$CaCl_2$ 0.05 mol.	$CaCl_2$ 0.125 mol.
mol.	*min.*	*min.*	*min.*
0	23	10	21
0.005	23	10	21
0.01	23	10	21
0.025	27	11	21
0.05	28	13	22
0.1	34	18	25
0.15	48	20	41
0.2	62	29	52
0.25	92	46	80
0.3	on the next morning coagulated	62	120
0.35	,,	79	on the next morning coagulated
0.4	,,	123	,,
0.45	non coagulable	147	,,
0.5	,,	201	non coagulable
1.0	,,	on the next morning coagulated	,,

TABLE IV.

$C_3H_4(OH)(COONa)_3$.

Concentration of salt added.	Time of coagulation.		
	$CaCl_2$ 0.03 mol.	$CaCl_2$ 0.05 mol.	$CaCl_2$ 0.08 mol.
mol.	*min.*	*min.*	*min.*
0	12	9	11
0.0001	12	9	11
0.0005	12	9	11
0.001	13	9	11
0.0025	14	9	10
0.005	15	9	10
0.0075	17	9	8
0.01	20	10	7
0.025	non coagul-able	18	8
0.05	,,	38	12
0.075	,,	non coagul-able	24
0.1	,,	,,	55
0.15	,,	,,	non coagul-able
0.2	,,	,,	,,
0.25	,,	,,	,,

TABLE V.

$BaCl_2$.

Concentration of salt added.	Time of coagulation.		
	$CaCl_2$ 0.035 mol.	$CaCl_2$ 0.05 mol.	$CaCl_2$ 0.125 mol.
mol. 0	*min.* 23	*min.* 10	*min.* 21
0.005	15	10	23
0.01	16	10	25
0.025	19	10	34
0.05	25	18	37
0.1	100	34	72
0.15	on the next morning coagulated	54	140
0.2	non coagulable	85	on the next morning coagulated
0.25	,,	on the next morning coagulated	,,
0.3	,,	,,	,,
0.35	,,	non coagulable	non coagulable
0.4	,,	,,	,,
0.45	,,	,,	,,
0.5	,,	,,	,,

TABLE VI.

$MgCl_2$.

Concentration of salt added.	Time of coagulation.		
	$CaCl_2$ 0.045 mol.	$CaCl_2$ 0.08 mol.	$CaCl_2$ 0.13 mol.
mol.	*min.*	*min.*	*min.*
0	12	10	16
0.005	12	10	16
0.01	13	11	16
0.025	14	12	17
0.05	17	16	28
0.1	33	30	77
0.15	100	91	330
0.2	on the next morning coagulated	290	on the next morning coagulated
0.25	non coagulable	on the next morning coagulated	,,
0.3	,,	non coagulable	non coagulated
0.35	,,	,,	,,
0.4	,,	,,	,,
0.45	,,	,,	,,
0.5	,,	,,	,,

TABLE VII.

$K_4Fe(CN)_6$.

Concentration of salt added.	Time of coagulation.		
	$CaCl_2$ 0.03 mol.	$CaCl_2$ 0.05 mol.	$CaCl_2$ 0.08 mol.
mol.	*min.*	*min.*	*min.*
0	12	9	11
0.0001	12	9	11
0.0005	12	9	11
0.001	12	9	11
0.005	12	9	11
0.01	12	9	11
0.025	17	16	25
0.05	50	47	67
0.075	118	274	on the next morning coagulated
0.1	incomplete coagulation	non coagulable	non coagulable
0.15	non coagulable	,,	,,
0.2	,,	,,	,,
0.25	,,	,,	,,

TABLE VIII.

$Al_2(SO_4)_3$.

Concentration of of salt added.	Time of coagulation.		
	$CaCl_2$ 0.06 mol.	$CaCl_2$ 0.09 mol.	$CaCl_2$ 0.13 mol.
mol.	*min.*	*min.*	*min.*
0	14	8	13
0.00005	14	8	13
0.0001	14	8	14
0.00025	14	8	14
0.0005	15	9	14
0.001	18	11	16
0.0025	non coagulable	26	32
0.005	,,	non coagulable	non coagulable
0.01	,,	,,	,,

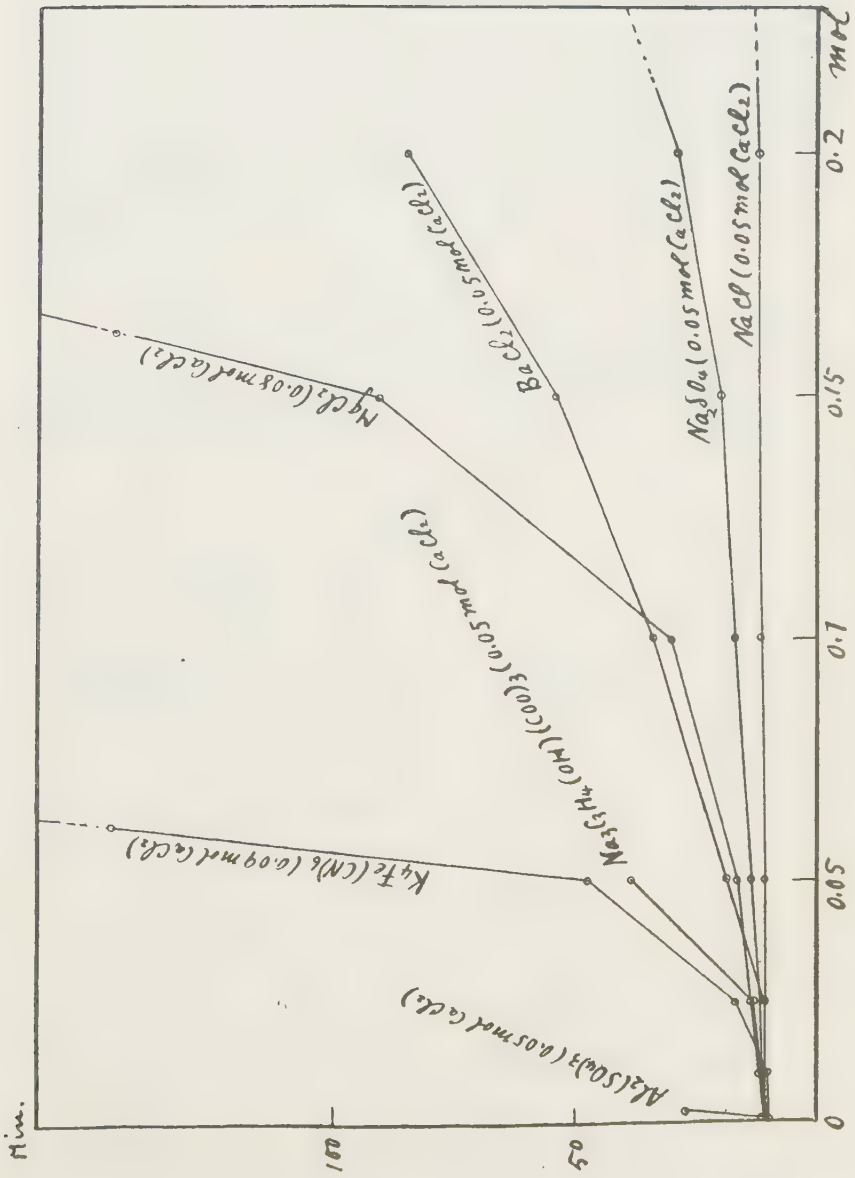
As can be seen from the table sodium chloride has only a slight effect on the coagulation time in a low concentration. Near 1 molecular solution the retardation is quite noticeable.

Sodium sulphate, barium and magnesium chloride all above 0.1 mol, and aluminium sulphate above 0.0025 mol retard the rate of coagulation. These effects seem probably be due to physicochemical change of fibrinogen by these salts. Oxalate naturally checks the coagulation by deprivation of calcium and inhibiting thus the formation of thrombin from prothrombin.

3. The effect of salt on coagulation of fibrinogen by thrombin.

The effect of various salts on coagulation of blood plasma in above mentioned experiments may be partly due to its action on the formation of thrombin from prothromin and partly

Fig. 1.


Min.
100
50
0
0.05
0.1
0.15
0.2
mM
$Al_2(SO_4)_3$ (0.05 mol $CaCl_2$)
$K_4Fe(CN)_6$ (0.09 mol $CaCl_2$)
$Na_3C_6H_5O_7$ (0.05 mol $CaCl_2$)
$MgCl_2$ (0.08 mol $CaCl_2$)
$BaCl_2$ (0.05 mol $CaCl_2$)
Na_2SO_4 (0.05 mol $CaCl_2$)
NaCl (0.05 mol $CaCl_2$)

due to that on fibrin-formation by thrombin. I have, therefore, studied also the effect of salt on coagulation of fibrinogen by thrombin.

Fibrinogen was prepared by the addition of the equal volume of saturated NaCl solution into the oxalate plasma, washed with a half saturated solution of sodium chloride and dissolved in a 1 per cent solution of sodium chloride. This

TABLE IX.

Time for coagulation.

Conc. of solution.	NaCl	$NaC_2H_3O_2$	Na_2SO_4	$Na_3C_3H_4(OH)(COO)_3$	$CaCl_2$	$BaCl_2$	$MgCl_2$
mol.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
0	11	11	11	11	11	11	11
0.05		10	15	25	8	11	13
0.1	11	10	19	32	10	11	16
0.15		11	22	34	12	13	21
0.2	11	11	24	36	14	16	28
0.25		12	26	37	18	20	33
0.3	11	13	28	37	25	26	58
0.35		12	29	36	30	32	82
0.4	13	12	30	28	39	41	120
0.45		12	30	23	48	58	
0.5	17	12	30	16	60	70	
0.6	19	12					
0.7	22	12					
0.8	25	12					
0.9	28	12					
1.0	33	12					

procedure was repeated three times. Such a preparation did not give a clot in twenty-four hours when calcified in the presence of kephalin. The concentration was nearly 0.29 %.

Thrombin solution. Horse serum was put into five times its volume of alcohol and precipitate was filtered and mixed with 5–6 volumes of abs. alcohol and filtered. Washing by this process was once more repeated. 2 gm. of this powder was shaken with 100 cc. and dist. water for three hours and centrifuged.

Experiment. The mixture of 2 cc. of fibrinogen solution, 1 cc. of thrombin solution and 1 cc. of salt solution of different consentration was put into the thermostat at 30°C. and the time for setting was observed. The result is shown in the following table.

TABLE X.

$K_4Fe(CN)_6$		$Al_2(SO_4)_3$	
Concentration of salts added.	Time.	Concentration of salts added.	Time.
mol.	*min.*	*mol.*	*min.*
0	15	0	17
0.0005	15	0.000001	17
0.001	16	0.0000025	17
0.0025	17	0.000005	17
0.005	20	0.00001	18
0.01	24	0.000025	18
0.02	38	0.00005	17
0.03	62	0.0001	14
0.04	81		
0.05	110		

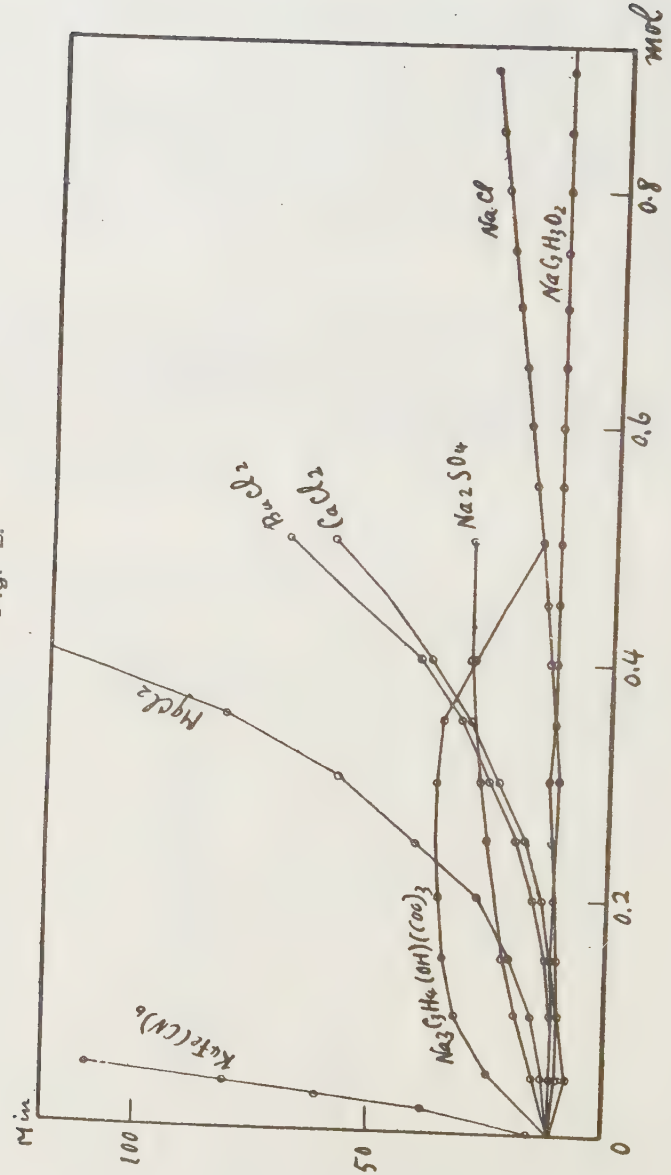

Min
100
50
0
0.2
0.4
0.6
0.8
mol
$MgCl_2$
$K_4Fe(CN)_6$
$Na_3C_3H_4(OH)(COO)_3$
$BaCl_2$
$CaCl_2$
Na_2SO_4
NaCl
$NaC_2H_3O_2$

Fig. 2.

As this table shows the effect of sodium chloride and sodium acetate was quite small, while $CaCl_2$, $BaCl_2$ and $MgCl_2$ retard the coagulation time. The matter with sodium citrate was especially noticeable, for the addition of this salt increased the time for complete coagulation at first and decreased again when the concentration of salt added became over 0.4 per cent. It is also remarkable that calcium accelerates the coagulation of fibrinogen by thrombin. That this is not due to the formation of thrombin from prothrombin contained in thrombin is verified by experiment, which will be mentioned far below. (See page 144).

II. The effect of H' Concentration.

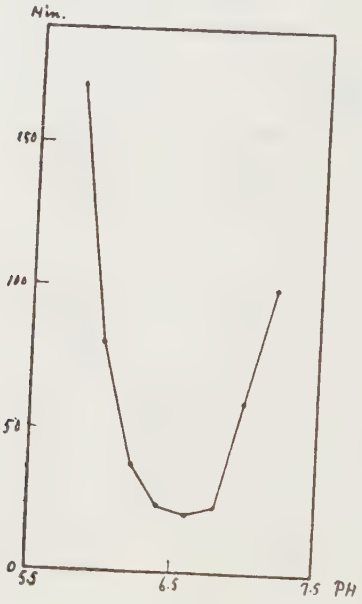
The effect of hydrogen ion concentration on the coagulation of fibrinogen by thrombin was studied by using phosphat-NaOH mixture after Clark as the buffer. To the mixture of 2 cc. of fibrinogen solution and 1 cc. of this buffer solution 1 cc. of thrombin solution was added and put into the thermostat at 30°C. The result is as follows.

TABLE XI. (See Fig. 3)

pH	Time for coagulation.
	min.
5.8	170
6.0	80
6.2	38
6.4	24
6.6	21
6.8	24
7.0	60
7.2	100
7.4	not coagulable
7.6	,, ,,

The most favorable acidity for the coagulation of fibrinogen is shown to be at pH=6.6.

Fig. 3.



III. The Influence of Temperature.

1. *The effect of heat on the stability of thrombin.*

5 cc. of thrombin solution were exposed for one hour to the varied temperature, ranging between −10° and 100°C each with interval of ten degree. 1 cc. of the thrombin solution thus treated was added to 2 cc. of fibrinogen solution and put into the thermostat at 30°C as usual. The time required to the complete coagulation is shown in the following table.

TABLE XII. (See Fig. 4)

Effect of temperature on the stability of thrombin.

Temperature.	Time.
	min.
—10° C	13
0	11
10	11
20	11
30	12
40	32
50	105
60	230
70	ca 7.5 hrs.
80	on the next morning coagulated
85	non coagulable
90	„ „
100	„ „

As the table indicates there is only little effect of heating on thrombin at body temperature, above which, however, it becomes quite noticeable increasing with the temperature. Lethal temperature seems to lie nearly at 80°C.

2. Influence of temperature on velocity of coagulation.

2 cc. of fibrinogen solution and 1 cc. of thrombin solution, both of which were previously maintained separately at varying temperature for 30 minutes, was mixed at the same temperature, and the time of onset of coagulation was noted. The result is as follows.

TABLE XIII. (See Fig. 5)

Effect of temperature on the velocity of coagulation.

Temperature.	Time.
0	6.5 hrs.
10	67 min.
15	36 ,,
20	24 ,,
25	16 ,,
30	11 ,,
35	9 ,,
40	8 ,,
45	12 ,,
50	incomplete coagulation

Fig. 4.

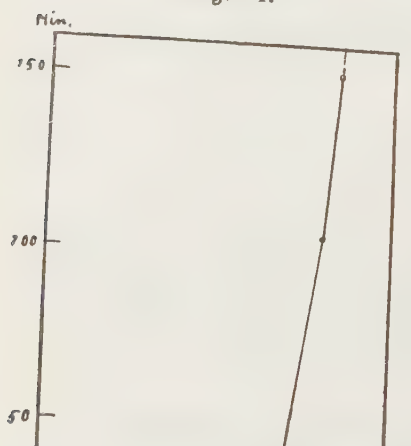
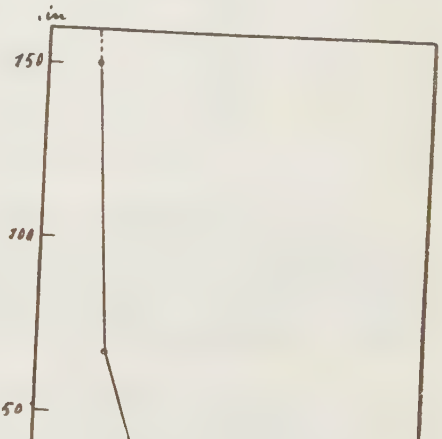


Fig. 5.



As we can see from the table the temperature coefficient for a range of 10°C. is quite large between 0°C. and 10°C. amounting over 10. Between 10° and 20°C. there is also a marked acceleration in the time of coagulation, temperature coefficient being approximately 2.8. When the temperature rises, the temperature coefficient becomes smaller, as it is 2.2 between 20° and 30C° but there is still an acceleration in the time of coagulation, the temperature coefficient being 1.4. These findings confirm those of Burker (1904) and Addis (1908). Beyond 40°C. the temperature coefficient becomes negative.

IV. Tests with Tissue Extract.

Preparation of lung extract. Calf lung was first freed from its bronchi and connective tissue and drawn through a meat-chopper. The paste was then spread over a glass plate and dried in Faust's drying apparatus. The dried mass was pulverised and driven through a sieve of fine mesh. Two grams of this powder were shaken with 100 cc. of distilled water and centrifuged. The upper solution was filtered and used for the experiment.

1. The influence of lung extract on the coagulation of plasma.

2 cc. of oxalated plasma were added to either 1 cc. of tissue extract or calcium chloride together with 1 cc. of water, or were added to both of these solutions and the time of setting of clot was observed. The result is shown on the following table.

TABLE XIV.

Mixture.	Coagulation time.
2 cc. plasma + 1 cc. extract + 1 cc. aqua dest	not coagulated.
2 cc. ,, + 1 cc. 0.1 m. $CaCl_2$ + 1 cc. ,,	12 min.
2 cc. ,, + 1 cc. ,, ,, + 1 cc. extract	2 min.

As this table indicates, the tissue extract had no influence upon the coagulation in the absence of $CaCl_2$, while in the presence of $CaCl_2$, it accelerated the time of coagulation a great deal.

2. The influence of tissue extract on the coagulation of fibrinogen solution by thrombin.

To the mixture of 2 cc. of fibrinogen solution and 2 cc. of thrombin solution was added either 1 cc. of lung extract or of pure water and their coagulation time was recorded. The result can seen in table XV.

TABLE XV.

Mixture.	Coagulation time.
2 cc. fibrinogen + 1 cc. aqua dest. + 2 cc. thrombin	14 min.
2 cc. ,, + 1 cc. lung extract + 2 cc. ,,	ca. 3 hrs.

Thus in the presence of lung extract the coagulation is retarded a great deal.

3. The influence of lung extract on the activation of prothrombin.

Preparation of prothrombin solution.—Oxalated plasma of horse was added with five times its volume of absolute alcohol. The resultant precipitation was allowed to stay until next morning, then the supernatant solution was decanted off and about the same quantities of fresh absolute alcohol were added. The precipitate was then filtered, washed with absolute alcohol and ether and dried in the air. Five grams of the precipitate thus obtained were shaken with 100 cc. of water and centrifuged. The supernatant part which contained the thrombogen, was used for the experiment.

Procedure.—The mixture of 3 cc. of fibrinogen solution and 1 cc. of thrombogen solution was added to either 1 cc. of extract or 1 cc. of 0.1 mol $CaCl_2$-solution or both of them, and placed in the thermostat of 30°C. As it will be shown in the following table, if any one of the 4 components (fibrinogen, prothrombin, tissue extract and $CaCl_2$) failed, there was no coagulation observable.

TABLE XVI.

Nature of mixture.	Coagulation time.
3 cc. fibrinogen + 3 cc. aqua dest.	not coagulated
3 cc. ,, + 1 cc. 0.1 m. $CaCl_2$ + 2 cc. aqua dest.	,, ,,
3 cc. ,, + 1 cc. extract + 2 cc. ,, ,,	,, ,,
3 cc. ,, + 1 cc. prothrombin + 2cc. ,, ,,	,, ,,
3 cc. ,, + 1 cc. ,, + 1 cc. extract + 1 cc. aqua dest.	,, ,,
3 cc. ,, + 1 cc. prothrombin + 1 cc. 0.1 m. $CaCl_2$ + 1 cc. aqua dest.	,, ,,
3 cc. ,, + 1 cc. prothrombin + 1 cc. extract + 1 cc. 1 m. $CaCl_2$	40 min.

To test the influence of tissue extract on the formation of thrombin from prothrombin, 2 series of experiments were performed. In the first series, A, $CaCl_2$, tissue extract and fibrinogen were mixed and put in a thermostat of 30C°. for 10 minutes and prothrombin solution was then added, in the other series, B, $CaCl_2$, tissue extract, and prothrombin solution were first mixed and put into the thermostat of 30°C. for 10 minutes and then added to fibrinogen solution. The result is put in the table XVII.

TABLE XVII.

Nature of mixture.	Time of coagulation.	
	A	B
3 cc. fibrinogen+1 cc. thrombogen+1 cc. ex.+1 cc. 0.1 m. $CaCl_2$	ca 1 hr.	16 min.
3 cc. fibrinogen+1 cc. thrombogen+1 cc. ex.+1 cc. 0.08 m. $CaCl_2$	50 min.	12 min.
3 cc. fibrinogen+1 cc. thrombogen+1 cc. ex.+1 cc. 0.06 m. $CaCl_2$	1 hr.	13 min.

We can see from the table that the tissue extract is necessary for the formation of the thrombin from prothrombin.

4. The influence of the concentration of $CaCl_2$ on the coagulation of fibrinogen by prothrombin and lung extract.

To find out the influence of concentration of $CaCl_2$, the following experiment was performed. 1 cc. of prothrombin solution, 1 cc of lung extract and 1 cc. of $CaCl_2$ of various concentration were mixed in test tubes and put into a thermostat at 30°C. for 10 minutes, and then 3 cc. of fibrinogen solution were added. The coagulation time is shown in the following table.

TABLE XVIII.

Concentration of $CaCl_2$ added.	Time of coagulation.	Concentration of $CaCl_2$ added.	Time of coagulation.
mol	*min.*	*mol*	*min.*
0	not coagulated	0.1	13
0.01	,,	0.12	15
0.02	22	0.14	18
0.04	12	0.16	21
0.06	11	0.18	25
0.08	12	0.2	50

The table shows that increase of $CaCl_2$ to a certain amount accelerates the time of coagulation. Beyond that point, however, retardation will be observed.

5. The preparation of active substance from the calf-lung.

1. The watery lung extract was shaken in a separating funnel with twice its volume of ether and both ethereal and watery part were tested in their activity on the activation of prothrombin. For this purpose two cc. of fibrinogen solution, .1 cc. of prothrombin solution and 0.4 cc. of 0.2 mol $CaCl_2$ were mixed and each part from lung extract was added. It was found out in this experiment that the watery part was quite active, while the ethereal part remained without any action. From this result the active substance seemed as if it did not dissolve in ether.

2. The watery extract of the lung powder was slightly acidified with hydrochloric acid and shaken with ether in a separating funnel. Each part was neutralized with sodium carbonate and was tested as in (1). The watery part was still active, while the ethereal part had no influence for the coagulation. Therefore we can see that the active substances do not move to ether from watery solutions, even though the reaction is acid.

3. The dried powder of lung above mentioned was added to five times its volume of absolute alcohol and shaken often. After 3 hours alcohol was decanted off. The powder was then added to 3 parts of absolute alcohol and 1 part of ether, and shaken often. After 3 hours the alcohol-ether was decanted off. The same treatment was repeated with the mixture of 1 part alcohol and 3 parts ether and then pure ether. These alcohol, alcohol-ether and ether portions were evaporated in vacuum and the residues were dissolved with distilled water. The powder which was treated as above was extracted by shaking with pure water, and filtered.

1 cc. of each solution thus obtained was mixed with 2 cc. of fibrinogen solution, 1 cc. of prothrombin solution and 1 cc. of 0.1 mol $CaCl_2$, and the time of coagulation was observed in a thermostat at 30°C. The result was as in the following table.

TABLE XIX.

Part of the extract.	Effect.
1. Alcohol soluble part	not coagulated
2. Alcohol-ether (3 : 1) solution part	,, ,,
3. Alcohol-ether (1 : 3) solution part	coagulated in next morning
4. Ether soluble part	in 50 min. coagulated
5. Waterly extract of the powder which was treated with alcohol and ether	in 7 min. coagulated

From the above table we can say that the active substance can be extracted with ether from the complete dried tissue powder, while it seems to be insoluble in alcohol.

4. Calf lung was ground to a fine paste by a mincing machine. The paste was added to five times its volume of 90 % alcohol, and after 24 hours the mixture was filtered. The residue was treated with 90 % alcohol and absolute alcohol successively in the same manner and was pulverized and sifted.

A. The powder thus obtained was shaken with ten times its volume of ether and filtered after 24 hours. The residue from this ethereal solution was dissolved in water and the solution was tested in its influence for the coagulation. The effect was negative.

B. A part of the alcohol which was used to remove the water was evaporated and the residue divided in two; the first was dissolved with pure water (1), the second was treated in a similar way after washing with ether (2).

When the alcohol was evaporated in vacuum it became turbid. The turbidity disappeared on addition of ether. The ethereal part was separated and evaporated. The residue of ether soluble portion was taken up in pure water.

The solutions thus obtained were tested in their influence on coagulation as above mentioned. The result was as follows.

TABLE XX.

Nature of extract.	Coagulation time.
Control	not coagulated in 2 hours.
Alcohol soluble (1)	47 min.
Alcohol soluble (2) washed with ether	not coagulated in 2 hours.
Ether soluble	15 min.

The result shows that ether is a very suitable solvent for the active substance in comparison with alcohol, but under certain conditions it is easily soluble also in alcohol.

5. The dried powder of lung above mentioned was added to absolute alcohol and evaporated on a water-bath, this treatment being repeated several times. The dry powder was extracted with ether, aceton, benzol, chloroform, petroleum ether and alcohol successively.

Each solvent was evaporated respectively. The residues were dissolved with pure water and the solutions were tested as in the above experiment. The result is shown in the following table.

TABLE XXI.

Nature of extract.	Coagulation time.
1. Control	not coagulated in 2 hours.
2. Watery extract of the powder, treated with ether five time	"

Nature of extract.	Coagulation time.
3. Ether soluble	5 *min.*
4. Benzol soluble	15
5. Chloroform soluble	20
6. Alcohol soluble	27
7. Petroleum ether soluble	5
8. Aceton soluble	not coagulated in 2 hours.

From the solubility we can conclude that the active substance belongs to the group of phospholipin, especially kephalin.

6. Calf lung was ground to a fine paste as above described, and was added to five times its volume of acetone and the whole left to stand for 24 hours. The acetone was changed for three times and the hardened material was separated from the acetone and pulverized. The dried powder thus obtained was shaken with ether for 2 hours and then with absolute alcohol for 3 hours. The ether, alcohol and a part of the acetone which used for drying were evaporated in vacuum separately. The residues were taken up in pure water and tested in the same way as the above experiment. The result was as shown in the following table.

TABLE XXII.

Nature of extract.	Effect.
2 cc. plasma + 0.5 cc. 0.2 % $CaCl_2$ + 1 cc. aq. dest.	no coagulation occurred
2 cc. ,, + 0.5 cc. ,, ,, + 1 cc. acetone soluble	,,
2 cc. ,, + 0.5 cc. ,, ,, + 1 cc. alcohol soluble	incompletely coagulated in 3 hours
2 cc. ,, + 0.5 cc. ,, ,, + 1 cc. ether soluble	coagulated in 15 min.

From the result we can see that the active substance is readily soluble in ether, while in acetone it is insoluble.

7. With 1 per cent solution of pure lecithin and kephalin which were obtained from ox-brain, the influence for the coagulation was tested as the following table indicates.

TABLE XXIII.

Nature of mixture.	Time of clotting.
2 cc. plasma + 0.5 cc. 0.2 % $CaCl_2$ + 1 cc. 1 % cephalin	25 min.
2 cc. „ + 0.5 cc. „ „ + 1 cc. lecithin	in 24 hrs. coagulated
2 cc. „ + 0.5 cc. „ „ + 1 cc. aq. dest.	not coagulated.

The table shows that kephalin has distinctly the quality to accelerate the coagulation of plasma, while lecithin has the same property only in a small degree. This capacity of lecithin may, probably, be due to a slight contamination of kephalin in it.

6. Experiment with the extract of lung which is freed from blood.

Under narcosis with ether and chloroform a rabbit was bled from A. carotis and the injection of 0.9% solution of NaCl into V. jugularis followed until the liquid which flowed out from carotis became colourless. The lung of the animal thus treated was taken out and ground to a fine paste as in the case of calf lung. The paste was evaporated on a water-bath under the addition of absolute alcohol several times, and dried completely. The dried powder was then rubbed up with ten times its weight of 0.9% solution of NaCl in a mortar. This watery extract was tested as in the following table.

TABLE XXIV.

Nature of mixture.	Time of clotting.
1 cc. plasma + 0.25 cc. 0.01 m. $CaCl_2$ + 1 gtt. 0.9 % NaCl	220 min.
1 cc. ,, + 0.25 cc. ,, ,, + 1 cc. waterly extract	16 min.

100 cc. of oxalated horse plasma was dialysed 2 times in collodion bag against 100 cc. 0.9% solution of NaCl, and then dried with Faust's drying apparatus at lower temperature. The dried mass was then dissolved in 2 times its original volume 0.9% NaCl solution and filtered. In the table XXIV and in the further experiments we used always the plasma thus treated.

1.0225 gm. of the dried powder of rabbit lung was extracted with ether. Evaporating off ether we obtained 0.205 grams of residue. The residue was dissolved in water to make solution of various concentrations and its influence on coagulation was studied. The result is shown in the following table.

TABLE XXV.

1 cc. plasma + 0.25 cc. 0.01 m. $CaCl_2$ + 1 gtt. etherextract of lung.

Conc. of ether extract.	Time of coagulation.
%	*min.*
0	270
1/1000	160
1/100	37
1/10	18
1	15

0.2 gm. of dried residue of the ether extract was dissolved out with alcohol, acetone and ether successively, and each extract was evaporated. Residues were emulsified with pure water and their influence on the coagulation was tested. The result was as follows.

TABLE XXVI.

1 cc. plasma + 0.25 cc. 0.01m. $CaCl_2$ + 1 gtt. of emulsion.

Conc. of extract.	Alcohol-soluble.	Acetone soluble.	Ether soluble.
%	*min.*	*min.*	*min.*
0	25	25	28
1/100	19	25	8
1/10	16	17	5
1	12	13	3

From tables XXIV—XXVI we can ascertain that the extract of rabbit lung which was freed from blood is able to accelerate the coagulation just as well as extract of calf lung and the ether is the most suitable solvent for the active substance.

7. *Experiment with the extract of leucocyte and erythrocyte.*

Preparation of the dried powder of leucocyte and erythrocyte.— 2000 cc. of horse blood was, after the addition of 3 grams of potassium oxalate, left to stand for 24 hours. Leucocytes were gathered in upper layer of precipitate, while erythrocytes formed the lower layer. Each layer was separated carefully and washed with 0.9% NaCl solution several times by means of a centrifuge. Both the leuco—and the erythrocytes thus obtained were dried completely by evaporation on addition of alcohol and pulverized. The dried powder was then extracted

with water or ether as in the case of lung. The ether extractive substance from 0.41 gram of leucocytes powder amounted to 0.04 gram, while that obtained from 18 grams of erythrocytes powder was only 0.2 gram.

The influence for coagulation was as follows.

TABLE XXVII.

Nature of mixture.	Coagulation time.
	nim.
1 cc. plasma + 0.25 cc. 0.01 m. $CaCl_2$ + 1 gtt. 0.9 % NaCl	220
1 cc. „ + 0.25 cc. „ „ + 1 gtt. 1 % watery extract of leucocyte	10
1 cc. „ + 0.25 cc. „ „ + 1 gtt. 1 % watery extract of erythrocyte.	16

TABLE XXVIII.

1 cc. plasma + 0.25 cc. 0.1 m. $CaCl_2$ + 1 gtt. ethereal extract of leucocyte or erythrocyte.

Conc. of ethereal extract.	Time of coagulation.	
	Leucocyte.	Erythrocyte.
%	*min.*	*min.*
0	270	270
1/1000	150	270
1/100	33	105
1/10	17	37
1	18	25

The ethereal extracts of leucocytes and erythrocytes were again extracted with acetone, alcohol and ether respectively and each extract was evaporated. The residues were emulsified with distilled water and their influence on coagulation was tested. The result was as shown in the following tables XVI. and XVII.

TABLE XXIX.

Extracts from leucocyte

1cc. plasma+0.25 cc. 0.01 m. $CaCl_2$+1 gtt. of emulsion.

Conc. of extract	Alcohol soluble	Acetone soluble	Ether soluble
%	*min.*	*min.*	*min.*
0	25	25	25
1/100	24	25	16
1/10	18	19	13
1	17	16	7

TABLE XXX.

Extracts from erythrocyte

1 cc. plasma+0.25 cc. 0.01 m. $CaCl_2$+1 gtt. of emulsion.

Conc. of extract	Alcohol soluble	Acetone soluble	Ether soluble
%	*min.*	*min.*	*min.*
0	25	25	25
1/100	20	25	17
1/10	16	23	14
1	8	13	7

The result shows that both the extracts of leucocytes and erythrocytes accelerate the coagulation of plasma, that of leucocytes being more intensive, and that the relation of the active substance for solvents is similar to that of lung.

8. Experiment with the emulsion of leucocyte and bloodplate.

Preparation of leucocyte-emulsion.—20 cc. of bouillon was injected into the peritoneal cavity of a guinea pig. After

4 hours a turbid liquid was obtained by laparotomy. The liquid was centrifuged and the precipitate was washed several times with 0.9% NaCl solution. After centrifugation (2500 rotations per minute) for 10 minutes the precipitate was rubbed in a mortar with 10 times its volume of 0.9% NaCl solution.

Preparation of bloodplate-emulsion.—Blood of a rabbit was centrifuged (2500 rotations for a minute) for 4 minutes. This supernatant clear part was centrifuged for 15 minutes, over again. The precipitate was washed several times and rubbed up in a mortar with 40 times its volume of 0.9% NaCl solution. The mulsions thus obtained were tested as follows.

TABLE XXXI.

Nature of mixture.	Time of coagulation.
	min.
1 cc. plasma + 0.25 cc. 0.01 m. $CaCl_2$ + 1 gtt. 0.9 % NaCl	220
1 cc. ,, + 0.25 cc. ,, ,, ,, emulsion of leucocyte	50
1 cc. ,, + 0.25 cc. ,, ,, ,, emulsion of blood plate	40

From this result we can see that both emulsion of leucocyte and bloodplate contain the active substance and the emulsion of bloodplate acts more intensively in spite of its smaller concentration—dilution being about fourfold when compared with the leucocyte emulsion.

V. Experiments with Kepharin.

Preparation of kephalin.—Fresh ox-brain was freed from serous membrane and vessels, and was ground to a fine paste by a mincing machine. The paste was added to five times its volume of acetone and left to stand for 12 hours and then the acetone was decanted off. The same process was repeated twice, and the hardened material was ground to a fine

powder by a mortar and sifted through a fine mesh sieve. The fine powder thus prepared was extracted with ether several times by means of a shaking machine. After filtration the extract was concentrated in vacuum until a turbidity appeared, and then were added five times its volume of acetone. The precipitate was separated from liquid by decantation and was dissolved in as little amount of chloroform as possible. The chloroform was then washed twice with the same volume of 1% NaCl solution and concentrated in vacuum until turbidity appeared and was added with five times its volume of acetone and precipitated. The precipitate was separated entirely from acetone and dissolved in a small amount of ether. The precipitation with acetone was repeated once more. The residue was dissolved in ether and then evaporated until the odour of ether vanished. The residue was dissolved in a small amount of ether, and then precipitated with five times its volume of alcohol. The precipitate was dissolved in a small amount of ether and the ethereal solution was dropped in a fine stream into the alcohol which was heated at 60°C. and it was decanted off. The precipitate was washed with acetone. The kephalin thus obtained was preserved in a coloured desiccator, and used for experiments.

I. The influence of kephalin on the coagulation of plasma.

A. After mixing 1 cc. of plasma, 1 gtt. kephalin of various concentrations and 0.25 cc. of 0.01 mol $CaCl_2$, the time of coagulation was observed. The result was as shown in the following table.

TABLE XXXII.

Conc. of kephalin added.	Time of coagulation.
0 %	*min.* 90
1/512	44

Conc. of kephalin added.	Time of coagulation.
%	*min.*
1/256	27
1/128	16
1/64	9
1/32	7
1/16	6
1/8	5
1/4	4.5
1/2	4.25
1	4.25

B. 2 cc. of horse serum were added to 2 cc. of distilled water as in (*A*), or 1 cc. of 1% kephalin solution and 1 cc. of distilled water as in (*B*). Solutions thus prepared were mixed with 0.8 cc. of plasma and the time of coagulation was observed. The result was as follows.

TABLE XXXIII.

2 cc. of horse serum + 2 cc. of aq. dest. ..... A.
2 cc. of horse serum + 1 cc. of 1% kephalin + 1 cc. of aq. dest... B.

0.8 cc. of plasma + X gtt of A or B + (16−X) gtt of aq. dest.

X	Time of coagulation.	
	A	B
	min.	*min.*
16	12	3
8	20	4
4	39	9
2	not coagulated	14
1	„	40

C. 30 cc. of horse serum were added to 2 cc. of 0.1 mol potassium oxalate to free from calcium, and then dialysed in a collodion bag against 700 cc. of 0.9% NaCl solution three times inside a refrigerator. The serum thus treated was mixed with 0.8 cc. of plasma, and 0.1 cc. of 1% kephalin or distilled water and the influence on the coagulation was observed. The result was as follows.

TABLE XXXIV.

0.8 cc. of plasma + X gtt. of serum + (8−X) gtt of aq. dest + 0.1 cc. of 1% kephalin or aq. dest

X	Time of coagulation.	
	1% kephalina dded.	Aq. dest added.
	min.	*min.*
8	60	60
6	110	110
4	260	260
2	no coagulation until next morning	incomplete coagulation

As the tables XXXII and XXXIII show, kephalin accelerates the coagulation of plasma, but its presence in too large an amount retards the coagulation. As the table XXXIV shows, kephalin accelerates the coagulation only in the presence of calcium.

II. The influence of temperature on the stability of kephalin.

1% solution of kephalin was heated in an autoclave at various temperature degrees for one hour and 1 drop of it was mixed with 1 cc. of plasma and 0.25 cc. of 0.01 mol $CaCl_2$, and the influence for coagulation was observed. The result was as shown in following table.

TABLE XXXV.

Temperature.	Time of coagulation.
	min.
Control	7
100°C	7
110	7
120	7
130	11
140	27
150	45

As the table shows, the temperature below 120°C. gives no influence on the stability of kephalin and the temperature above 130° reveals some influence on it, but even the kephalin which was treated in an autoclave at 150°C. for one hour still accelerated the time of coagulation.

III. The effect of chemical treatments on the stability of kephalin.

1. A strong current of hydrogen was led through 1% ethereal solution of kephalin under catalytic action of platin black for 24 hours, until the solution did not absorb iodine at all. The solution thus treated was evaporated in vacuum and the residue was emulsified with 100 times its volume of distilled water. The influence of this solution for the coagulation was observed. As the following table shows, the accelerating action of kephalin on coagulation was not changed by hydrogenation.

TABLE XXXVI.

Mixture.	Time of coagulation.
	min.
0.8 cc. plasma + 0.1 cc. 0.1 m. $CaCl_2$ + 0.1 cc. aq. dest	22
0.8 cc. „ + 0.1 cc. „ „ + 0.1 cc. 1% kephalin	3
0.8 cc. „ + 0.1 cc. „ „ + 0.1 cc. 1% hydrogenated kephalin	3

2. 10 cc. of 1% watery solution of kephalin were shaken with 3 times its volume of Van Slyke's reagent (the mixture of 12 parts of 30% $NaNO_2$ and 3 parts of glacial acetic acid) in a separating funnel. The whole was evaporated on a water-bath. The residue was extracted with petroleum ether and the extract was evaporated after it had been washed several times with pure water. The residue thus obtained, 0.038 gm. was dissolved in 3.8 cc. of distilled water. The solution did not produce any trace of nitrogen by the action of Van Slyke's reagent. (Solution A).

3. 40 cc. of 1 per cent kephalin solution was added to 120 cc. of formalin and shaken strongly. The whole was then evaporated and its residue was extracted with petroleum ether. The extract was again evaporated. Extraction and evaporation were repeated again. The last petroleum ether extract was washed and evaporated as in 2. The residue thus obtained amounted to 0.114 gm. and was emulsified with 11 cc. of distilled water. The emulsion did not produce any traces of nitrogen by the action of Van Slyke's reagent. (solution B).

The influence of the solution A and B on the coagulation was observed as follows.

TABLE XXXVII.

Mixture.	Time of coagulation.
	min.
1 cc. plasma + 0.25 cc. 0.01 m. $CaCl_2$ + 1 gtt. aq. dest.	50
1 cc. „ + 0.25 cc. „ „ + 1 gtt. solution A.	47
1 cc. „ + 0.25 cc. „ „ + 1 gtt. solution B.	40
1 cc. „ + 0.25 cc. „ „ + 1 gtt. 1% kephalin.	17

As the table shows, the influence of kephalin for the coagulation was reduced distinctly by the treatment of nitrous acid or formalin. The product of formalin treatment became hardly soluble in water.

4. The influence of hydrolysis.

a. After the method of Parnas, kephalin was heated in an autoclave for twelve hours at 120°C. with barium hydrate. The product of hydrolysis, thus obtained, had no influence for coagulation.

b. To 50 cc. of 5% ethereal solution of kephalin were added 25 cc. of alcoholic solution of sodium hydroxide and to stand at about 30°C. for 1, 2, 3, 5 or 24 hours with occassional shakings. The alcoholic solution was prepared by the addition of 100 cc. of absolute alcohol to 6 cc. of saturated solution of sodium hydroxide. The unsaponified part of each partial saponification was taken away by adding water. The ethereal parts were neutralized with hydrochloric acid and washed with distilled water several times, and then evaporated.

One part of the residue was dissolved with a small amount of ether and rubbed up in a mortar with 100 times its weight of pure water. With 2 drops of emulsions thus obtained the influence for the coagulation was observed as in the above cases. The other part of residue was used for the determination of nitrogen and phosphorus. The result is as shown in the following table.

TABLE XXXVIII.

	Nitrogen	Phosphorus	N : P	Time of coagulation
Kephalin not treated				*min.* 6
hydrolysed for 1 hr.	0.227	0.511	1:1.01	25
for 2 hrs. ,,	0.158	0.384	1:1.01	30
for 3 hrs. ,,	0.242	0.624	1:1.03	30
for 5 hrs. ,,	0.342	0.467	1:1.17	35
for 24 hrs. ,,	0.213	0.156	1:0.62	90
control				90

The result shows, that the unsaponified residue of kephalin by partial sodium hydrolysis still accelerates somewhat the coagulation, so long as the proportion of N and P remains like that of kephalin. When, however, this relation was disturbed it lost its activity upon the coagulation. This action of the residue of partial hydrolysis may perhaps be due to residue of kephalin.

IV. The influence of substances which composed kephalin upon the coagulation of plasma.

1. *The influence of acidum palmnitinum.* One drop of sodium palmitate of various concentrations was mixed with 1 cc. of plasma and 0.25 cc. of 0.01 m. $CaCl_2$, and its influence for the coagulation in presence or absence of kephalin was observed. The result was as indicated in following table.

TABLE XXXIX.

Conc. of palmitinate.	Time of coagulation when concentration of kephalin is					
	0	$\frac{1}{10000}$%	$\frac{1}{1000}$%	$\frac{1}{100}$%	$\frac{1}{10}$%	1%
%	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
0	250	250	80	22	14	17
1/10000	250	250	80	22	14	17
1/1000	250	250	80	22	14	17
1/100	250	250	80	22	14	17
1/10	180	180	80	22	14	17

As the result shows acidum palmitinum accelerates the time of coagulation only when it is present in a large amount, the concentration less than 1/100% being without any influence for coagulation.

2. *The influence of the mixed unsaturated fatty acids, composed especially of linolic acid.* 0.1 cc. of nearly 1% solution sodium salt of unsaturated fatty acids mixture which was obtained from oleum lini, was added with 1 cc. of plasma and 0.25 cc. of 0.01 mol $CaCl_2$, and the time of coagulation was observed in presence or absence of kephalin. The result was as follows.

TABLE XXXX.

1 cc. plasma + 1 gtt. kephalin solution + 0.1 cc. aq. dest + 0.25 cc. 0.01 m. $CaCl_2$.....A.

1 cc. „ + 1 „ „ „ + 0.1 cc. sodium salt of fatty acid + 0.25 cc. 0.01 m. $CaCl_2$.....B.

Conc. of kephalin.	A	B
%	*min.*	*min.*
0	160	150
1/10000	150	150
1/1000	80	80
1/100	24	25
1/10	13	13
1	16	15

As the table shows, the mixture of the unsaturated acids, especially linolic acid, do not give any influence for the coagulation, at least, when used in the amount above mentioned.

3. *The influence of acidum stearicum.* The alcoholic solution of stearic acid was neutralised with sodium hydroxide and its influence for the coagulation was observed. The result is given in the following table.

TABLE XXXXI.

Mixture.	Time of coagulation.
	min.
1 cc. plasma + 1 cc. aq. dest + 0.25 cc. 0.01 m. $CaCl_2$.	16
1 cc. „ + 1 cc. 1/10000 % stearate + 0.25 cc. 0.01 m. $CaCl_2$	15
1 cc. „ + 1 cc. 1/1000 % „ + 0.25 cc. „ „	14
1 cc. „ + 1 cc. 1/100 % „ + 0.25 cc. „ „	11
1 cc. „ + 1 cc. 1/10 % „ + 0.25 cc. „ „	9

As the table shows, the stearate accelerates the coagulation of plasma distinctly.

4. *The influence of acidum oleicum.* The alcoholic solution of oleic acid was neutralised with sodium hydroxide and its influence for coagulation was observed, with the result indicated as follows.

TABLE XXXXII.

Nature of mixture.	Time of coagulation.
	min.
1 cc. plasma + 1 cc. aq. dest + 0.25 cc. 0.01 m. $CaCl_2$	20
1 cc. „ + 1 cc. 1/10000 % oleate + 0.25 cc. 0.01 m. $CaCl_2$	19
1 cc. „ + 1 cc. 1/1000 % „ + 0.25 cc. „ „	17
1 cc. „ + 1 cc. 1/100 % „ + 0.25 cc. „ „	15
1 cc. „ + 1 cc. 1/10 % „ + 0.25 cc. „ „	13
1 cc. „ + 1 cc. 1 % „ + 0.25 cc. „ „	12

As the table shows, oleate accelerates the time of coagulation in some degree.

5. *The influence of glycerophosphoric acid.* The influence of sodium glycerophosphate for coagulation was also studied.

TABLE XXXXIII.

Mixture.	Time of coagulation.
	min.
1 cc. plasma + 1 cc. aq. dest.	20
1 cc. „ + 1 cc. 1/10000% glycerophosphate	18
1 cc. „ + 1 cc. 1/1000% „	17
1 cc. „ + 1 cc. 1/100% „	20
1 cc. „ + 1 cc. 1/10% „	44
1 cc. „ + 1 cc. 1% „	not coagulated

As the table shows, the little amount of glycerophosphate accelerates somewhat the time of coagulation, but in its larger amount a distinct retardation is observed.

6. *Influence of aminoethylalcohol.* Aminoethylalcohol was neutralized with hydrochloric acid and its action on the coagulation was studied with the following results.

TABLE XXXXIV.

Mixture.	Time of coagulation.
	min.
1 cc. plasma + 1 cc. aq. dest + 0.25 cc. 0.01 m. $CaCl_2$	170
1 cc. „ + 1 cc. 1/10000% aminoethylalcohol + 0.25 cc. 0.01 m. $CaCl_2$	168
1 cc. „ + 1 cc. 1/1000% „ + 0.25 cc. 0.01 m. $CaCl_2$	160
1 cc. „ + 1 cc. 1/100% „ + 0.25 cc. 0.01 m. $CaCl_2$	165
1 cc. „ + 1 cc. 1/10% „ + 0.25 cc. 0.01 m. $CaCl_2$	160

As the table shows, aminoethylalcohol has no influence for coagulation.

7. *The influence of colamin-oleate.* The mixture in the equivalent amount of aminoethylalcohol and oleic acid was tested as in the following table.

TABLE XXXXV.

Mixture.	Time of coagulation.
	min.
1 cc. plasma + 1 cc. aq. dest + 0.25 cc. 0.01 m. $CaCl_2$	16
1 cc. ,, + 1 cc. 1/10000% aminoethylalcohol-oleic acid + 0.25 cc. 0.01 m. $CaCl_2$	15
1 cc. ,, + 1 cc. 1/1000% Aminoethylalcohol-oleic acid + 0.25 cc. 0.01 m. $CaCl_2$	15
1 cc. ,, + 1 cc. 1/100% ,,	13
1 cc. ,, + 1 cc. 1/10% ,,	12

As the table shows, colamin-oleate has a slight influence for the coagulation.

8. *The influence of aminoethylalcohol-stearic acid.* The mixture of aminoethylalcohol and acidum stearicum in equivalent amount was tested as in the following table.

TABLE XXXXVI.

Mixture.	Time of coagulation.	
	Plasma I	Plasma II
	min.	*min.*
1 cc. plasma + 1 cc. aq. Dest + 0.25 cc. 0.01 m. $CaCl_2$	68	170
1 cc. ,, + 1 cc. 1/10000% aminoethylalcohol stearic acid + 0.25 cc. 0.01 m. $CaCl_2$	44	123
1 cc. ,, + 1 cc. 1/1000% ,,	37	99
1 cc. ,, + 1 cc. 1/10% ,,	28	65

As the table shows, the mixture of aminoethylalcohol and stearic acid distinctly accelerates the time of coagulation. While all substances hetherto tested could not shorten the time

of coagulation less than half an amount, time of coagulation was minimised to about one-third.

VI. The Influence of the other Substances on the Coagulation.

1. The influence of cholesterin.

0.0923 gm. of cholesterin was dissolved in a small amount of ether, and rubbed up with 9 cc. distilled water. 0.1 cc. of this cholesterin emulsion was mixed with 1 cc. of plasma and 0.25 cc. of 0.01 mol $CaCl_2$ and its influence for the time of coagulation was observed, either in presence or absence of 1 gtt. of kephalin of various concentrations. The result was as follows.

TABLE XXXXVII.

1 cc. plasma + 1 gtt. kephalin + 0.25 cc. 0.01 m. $CaCl_2$ + 0.1 cc. of 1% cholesterin or aq. dest.

Conc. of kephalin.	With cholesterin.	Without cholesterin.
%	*min.*	*min.*
0	160	180
1/10000	160	180
1/1000	78	80
1/100	21	23
1/10	13	14
1	14	15

As the table shows, it seems as if cholesterin does not give any influence for the coagulation.

2. The influence of acidum cholicum.

Acid. cholic. was dissolved in equivalent amount of n/10 NaOH and the solution of any dilution prepared with 0.9% solution of natrium chloride. The action of choleate was tested as in I. The result is indicated as follows.

TABLE XXXXVIII.

1 cc. plasma + 1 gtt. kephalin + 0.1 cc. choleate + 0.25 cc. 0.01 m. $CaCl_2$

Conc. of choleate	Time of coagulation when concentration of kephalin was				
	0	1/1000 %	1/100 %	1/10 %	1 %
%	*min.*	*min.*	*min.*	*min.*	*min.*
0	28	19	7	6	4
1/10000	28	19	8	6	4
1/1000	28	19	9	6	4
1/100	28	19	8	6	4
1/10	28	19	8	7	4
1	32	25	11	11	11

The table indicates that choleate retards the coagulation only in a somewhat concentrated solution.

To study the influence of concentrated choleate solution on the coagulation more thoroughly the time of coagulation was measured both in the case of 1 cc. of 1/2% choleate solution with 1 gtt. kephalin of various concentrations, and in the case of 1 gtt. 1% kephalin with 1 cc. choleate of various concentrations. The result was as in the following tables.

TABLE XXXXIX.

1 cc. plasma + 1 cc. $\frac{1}{2}$% acid choleate + 1 gtt. kephalin + 0.25 cc. 0.01 m. $CaCl_2$

Conc. of kephalin.	Time of coagulation.
%	*min.*
0	50
1/1000	47
1/100	43
1/10	37
1	27

TABLE L.

1 cc. plasma+1 cc. choleate+1 gtt. 1% kephalin+0.25 cc. 0.01 m. $CaCl_2$

Conc. of choleate.	Time of coagulation.
0 %	14 min.
1/10	17
1/4	27
1/2	37
1	150

From the result, we confirm that choleate in a great amount retards the coagulation both in presence and absence of kephalin, while the small amount of it has only a weak influence.

3. The influence of albumin.

The crystallized albumin obtained from eggs was freed from salt by dialysis. 1 cc. of albumin solution of various concentrations was mixed with 1 cc. of plasma and 0.25 cc. of 0.1 mol $CaCl_2$, and the time of coagulation was determined in presence and absence of kephalin solution of various concentrations. The result was as follows.

TABLE LI.

1 cc. plasma+1 gtt. kephalin+0.25 cc. O. P. 1 mol $CaCl_2$+1 cc. albumin or 0.9% $NaCl_2$.

Conc. of Albumin.	Time of coagulation when concentration of kephalin was				
	0	1/1000 %	1/100 %	1/10 %	1 %
0 %	23 min.	16 min.	10 min.	9 min.	7 min.
1/32	16	31	19	6	4
1/16	20	32	8	6	4
1/8	22	30	12	7	4
1/4	19	25	17	10	4
1/2	16	25	15	10	5
1	8	16	12	11	6

Viewed from the result, albumin, in an increasing amount, first retards, and then accelerates the coagulation in the absence or presence of a minute quantity of kephalin. On the contrary, when the amount of kephalin is larger, the presence of albumin first accelerates and then retards the coagulation.

4. The influence of the extract of leech (Hirudin).

Preparation of the extract of leeches.—1000 heads of leeches were rubbed up in a mortar with glass granules. The paste was extracted with 700 cc. of pure water in a refrigerator for 24 hours. To facilitate the extraction the whole was shaken quite often. The extract was freed from mucinous matter by the acidulation with 2% acetic acid, neutralized with Na_2CO_3, and was evaporated at a low temperature. The residue was dried in a desiccator.

1 cc. of hirudin of various concentrations was mixed with 1 cc. of plasma, and 0.25 cc. of 0.01 mol $CaCl_2$ and its influence for coagulation was observed in presence or absence of kephalin. The result was as follows.

TABLE LII.

1 cc. plasma + 1 gtt. kephalin + 0.25 cc. 0.01 m. $CaCl_2$ + 1 cc. hirudin or 0.9% NaCl.

Conc. of hirudin.	Time of coagulation when concentration of kephalin was				
	0	1/1000 %	1/100 %	1/10 %	1 %
%	*min.*	*min.*	*min.*	*min.*	*min.*
0	125	55	25	16	12
1/50000	125	56	29	16	12
1/5000	130	60	30	17	12
1/500	465	220	65	28	16
1/50	not coag.	not coag.	365	120	52
1/5	,,	,,	not coag.	ca. 15 hrs.	ca. 11 hrs.

50 cc. of rabbit blood were mixed with 1 cc. of 10% leech-extract solution. With the hirudin plasma, thus obtained, the test the same as in the former experiment was repeated and the result as in the following table was obtained.

TABLE LIII.

1 cc. plasma + 1 gtt. kephalin + 0.25 cc. 0.01 m. $CaCl_2$ + 1 cc. hirudin-plasma or 0.9% NaCl.

Conc. of hirudin plasma.	Time of coagulation when concentration of kephalin was				
	0	1/1000 %	1/100 %	1/10 %	1 %
%	*min.*	*min.*	*min.*	*min.*	*min.*
0	75	52	26	14	13
0.1	135	65	40	18	14
1	300	230	95	37	16
10	not coag.	not coag.	ca 24 hrs.	60	27
100	,,	,,	not coag.	not coag.	ca. 9 hrs.

As the tables LII and LIII show, the extract of leech distinctly retards the coagulation of plasma and kephalin has the ability to neutralize the action of hirudin as already known.

In the further experiments, to the hirudinplasma was added only kephalin or $CaCl_2$ of various concentrations. We could assume that kephalin of a certain concentration was able to make the hirudin plasma coagulable, while $CaCl_2$ did not indicate such an ability.

5. The influence of peptone.

In the same manner as in the case of hirudin, the influence of peptone solution on the coagulation was studied, the result of this being given in the following table.

TABLE LIV.

1 cc. plasma + 1 gtt. kephalin + 1 cc. peptone + 0.25 cc. 0.01 m. $CaCl_2$

Conc. of peptone.	Time of coagulation when concentration of kephalin was				
	0%	1/1000%	1/100%	1/10%	1%
%	*min.*	*min.*	*min.*	*min.*	*min.*
0	58	22	16	10	6
1/1000	63	25	17	10	6
1/100	87	23	16	10	6
1/10	94	28	21	10	6
1	183	28	13	8	5

The table indicates that peptone added to the blood plasma in vitro retards the coagulation only when it is used in a large amount, the small quantity of it giving no influence. In the presence of plenty of kephalin a large amount of peptone rather seems to accelerate the coagulation.

VII. Experiment with Peptone Plasma.

Peptone plasma was obtained as usual by injection 10 cc. of 5% solution of peptone per kilogram of the weight of dog.

1. The acidity of peptone plasma.

The acidity of the peptone plasma was measured by the electrometric method. The result was not distinguishable from a common plasma, it giving a value of pH=7.6.

2. The influence of peptone plasma on the coagulation of plasma.

1 cc. of peptone plasma was added to 1 cc. of plasma, 0.25 cc. of 0.01 mol $CaCl_2$ and 1 drop of kephalin of various concentrations and the time of coagulation was determined. The result was as shown in the following table.

TABLE LV.

1 cc. plasma + 1 gtt. kephalin + 1 cc. peptoneplasma + 0.25 cc. 0.01 m. $CaCl_2$

Conc. of peptone plasma.	Time of coagulation when concentration of kephalin was				
	0	1/1000%	1/100%	1/10%	1%
%	min.	min.	min.	min.	min.
0	57	33	24	13	8
0.1	38	30	23	12	8
1	69	31	20	10	5
10	270	180	30	14	9
100	not coag.	not coag.	not coag.	17	9

From the table it seems that the peptone plasma somewhat accelerates to a small extent, while the great amount of it retards the coagulation distinctly.

3. The influence of trypsin on the peptone plasma.

Trypsin which was prepared after the method of Mays, was dissolved in various concentrations. 1 cc. of each solution was mixed with 1 cc. peptone plasma, either alone or with addition of 1 drop of kephalin solution of 1% concentration. The whole was put into a thermostat at 30°C. and the time of coagulation was observed. The result was as follows.

TABLE LVI.

1 cc. peptone plasma + 1 cc. trypsin + 1 gtt 1% kephalin or aq. dest.

Concentration of trypsin added.	Without kephalin.	With kephalin.
%		
0	not coagulated	not coagulated
1/1024	,,	incompletely coagulated
1/512	,,	in 24 hrs. coagulated
1/256	,,	,, ,,

Concentration of trypsin added.	Without kephalin.	With kephalin.
1/128 %	not coagulated	in 24 hrs. coagulated
1/64	,,	140 *min.*
1/32	,,	100 ,,
1/16	,,	90 ,,
1/8	,,	65 ,,
1/4	,,	60 ,,
1/2	,,	not coagulated
1	,,	,,
2	,,	,,

From the result we can presume that trypsin digests a part of the hindering substance and lets cephalin easily coagulate the mixture. Accordingly the peptone plasma may become coagulable. If, however, the effect of tripsin is too strong, the fibrinogen may be also digested and in this case the peptone plasma does not coagulate.

4. Only kephalin or $CaCl_2$ of various concentrations was added to peptone plasma, but the substances could not produce the coagulation.

VIII. Experiment with Prothrombin.

Influence of the extraction with alcohol and ether on the stability of thrombin powder.

200 cc. of horse serum was precipitated with 1000 cc. of absolute alcohol and the supernatant part was filtered off. The precipitate was added to three times its volume of absolute alcohol and the whole left to stand for 15 hours and was filtered. A part of the precipitate was added to some ether and dried in the air (thrombin A). The other part was shaken with five times its volume of absolute alcohol and was filtered. The same process was repeated with the mixture

of 2 parts of absolute alcohol and 1 part of ether, with the mixture of the same volume of alcohol and with ether and then with pure ether. The powder thus obtained was extracted moreover with warm ether by Soxleht's apparatus for 10 hours, and dried in the air. (thrombin B).

2 grams of thrombin A and B were shaken with 100 cc. of distilled water for 2 hours respectively, and centrifuged. The supernatant clear part was used for experiment.

With the solutions of thrombin A and B the tests were performed as indicated in the following table.

TABLE LVII.

Mixture.	Time of coagulation.
	min.
2 cc. fibrinogen solution + 1 cc. thrombin A.	17
2 cc. „ „ „ „ B.	17

As the table shows, the thorough extraction, with alcohol and ether causes no influence on the activity of thrombin.

1 cc. of thrombin B solution was added with 2 cc. of fibrinogen solution and X cc. of 1% kephalin and (1–X) cc. of distilled water and the influence of kephalin on the coagulation was observed. The result was as table II shows.

TABLE LVIII.

X	Time of coagulation.
	min.
0	17
0.1 cc.	17
0.2 cc.	17
0.4 cc.	17
0.8 cc.	18
1 cc.	21

From the result we can assume that the kephalin is not necessary for the coagulation of fibrinogen by thrombin, while the great amount of it rather retards the coagulation.

The influence of the addition of 1 cc. of $CaCl_2$ of various concentrations to the mixture of 2 cc. fibrinogen solution, 1 cc. thrombin solution and 1 gtt 1% kephalin was then studied and the following result was obtained.

TABLE LIX.

Conc. of $CaCl_2$ added.	Time of coagulation.
mol	*min.*
0	14
0.01	14
0.02	12
0.04	9
0.06	8
0.08	8
0.1	10

As the table shows, $CaCl_2$ in a certain concentration accelerates somewhat the coagulation. This phenomenon caused the doubt that the prothrombin may be found mixed with thrombin. To clear the question, we tried the next experiment.

XI. *Experiment with the thrombin which was freed from prothrombin.*

Preparation of thrombin solution.—100 cc. of horse serum was added with 5 cc. of 1% kephalin, and left to stand in a thermostat of 35°C. for 40 minutes. The serum was then added to 1000 cc. of 90% alcohol and after 15 hours the liquid part was filtered off. The precipitate was treated with absolute alcohol for 24 hours and during the time the alcohol

was renewed 3 times. The dried precipitate was shaken with the mixture of absolute alcohol and ether in equal part, and of 1 part of absolute alcohol and 3 parts of ether, and then pure ether, each for 3 hours. The precipitate was extracted moreover with ether by the Soxleht's apparatus for 8 hours. The solution of the powder thus obtained, which can be assumed as kephalin-free, was prepared and used as in the former test.

1. The influence of $CaCl_2$.

The mixture of 1 cc. of the thrombin solution, 1 cc. of fibrinogen solution and 1 cc. of $CaCl_2$ of various concentrations was put into a thermostat at 30°C. and the time of coagulation was observed.

In the second series of this experiment the mixture was added to 1 drop of 1% kephalin. The result was as in the following table.

TABLE LX.

Conc. of $CaCl_2$ added.	Time of coagulation.	
	Without kephalin.	With kephalin.
mol	*min.*	*min.*
0	13	14
1/256	12	13
1/128	11	12
1/64	10	11
1/32	12	12
1/16	12	13
1/8	20	20
1/4	38	38
1/2	240	240
1	not coagulated	not coagulated
		30°C.

The result indicates that $CaCl_2$ of a certain concentration accelerates somewhat the fibrinogen coagulation by thrombin also in absence of thrombogen.

2. The influence of kephalin.

1 cc. of thrombin solution, 1 cc. of fibrinogen solution and 1 cc. of kephalin of various concentrations were mixed, and the time of coagulation was observed in a thermostat at 30°C. As the following table shows we got the same result as in the former experiment with the thrombin, which was extracted.

TABLE LXI.

Conc. of kephalin added.	Time of coagulation.
%	*min.*
0	13
1/256	13
1/128	13
1/64	13
1/32	13
1/16	13
1/8	13
1/4	15
1/2	17
1	21
	30°C.

3. The influence of albumin, peptone, choleate and hirudin.

1 cc. albumin, peptone, choleate or hirudin of various concentrations were added with 1 cc. of fibrinogen solution and 1 cc. of thrombin solution, and the influence for coagulation was observed in a thermostat at 30°C. The result was as in the following table.

TABLE LXII.

The influence of albumin, peptone, choleate and hirudin on the time of coagulation.

Conc. of solution added.	Albumin.	Peptone.	Choleate.	Hirudin.
%	*min.*	*min.*	*min.*	*min.*
0	13	13	13	13
1/10000	13	13	13	14
1/1000	13	13	13	20
1/100	13	13	14	31
1/10	12	13	19	in 24 hours coagulated
1	11	23	210	not coagulated

The table indicates that in presence of a large amount albumin accelerates somewhat, while peptone and choleate retard the coagulation of fibrinogen solution by thrombin. Hirudin retards it distinctly as in the case of the plasma coagulation.

Summary.

1. The special effect of calcium for the activation of prothrombin into thrombin is shared by strontium.

2. Calcium accelerates the coagulation of blood not only through the activation of prothrombin into thrombin, but by promoting the formation of fibrin from fibrinogen by thrombin.

3. Thrombin is stable at the temperature below the body temperature. Its activity is maximum at body temperature.

4. The identification of tissue thromboplastic substance with kephalin was confirmed.

5. Kephalin maintains its thromboplastic character in full by the heating below 120°C. At higher temperature the activity is partly decreased.

6. The hydrogenation has no effect on the thromboplastic activity of kephalin.

7. The treatment of kephalin by nitrous acid or formalin causes the decrement of activity of kephalin as thrombokinase.

8. By the complete hydrolysis kephalin lost its thromboplastic activity. Out of its decomposition products glycerinphosphoric acid and aminoethylalcohol reveal no thromboplastic action. Stearate and aminoethylstearate especially the latter, promote, however, the coagulation of fibrinogen a great deal.

9. Cholesterol exerts no influence upon the coagulation of plasma. Cholic acid in a great amount retards the coagulation both in presence and absence of kephalin.

10. The influence of albumin in different concentrations on the velocity of coagulation in the absence and presence of kephalin in varying amount enveals a complicate, but regular relation, which needs a further study.

11. The treatment of peptone plasma with trypsin induces the coagulation, the rate increasing up to a certain point with the concentration of trypsin. This effect seems to lie in the hydrolysis of some inhibiting substance by trypsin. Further increase in trypsin inhibit however the coagulation, possibly by its hydrolytic action on fibrinogen.

12. Kephalin has no effect on the coagulation of fibrinogen by thrombin but in a large excess it tends to decrease the coagulation velocity.

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ÜBER DIE SPALTUNG DER STÄRKE DURCH AMYLASE. I.

Von

KEN HARADA.

(*Aus dem Biochemischen Laboratorium, Kaiserliche Universität. Tokyo Vorstand: Prof. Dr. S. Kakiuchi.*)

(Eingegangen am 7. Januar 1923)

I. Einleitung.

Über die Natur der Amylasen behauptete man von früher Zeit, dass sie aus zwei Arten von Enzymen bestehen, während auch die gegenteilige Behauptung, welche auf Einheit der Enzyme bestand, nicht fehlte. Obwohl beide Hypothesen lange Zeit eine Streitfrage bildeten, glaubt man heutzutage doch hauptsächlich an die Richtigkeit der ersten Annahme. Hiernach unterscheidet man nämlich in Amylase wenigstens zwei Enzyme, wovon das eine die Stärke zum Dextrin und das andere dieses weiter zur Maltose spaltet. Die Diastase wurde zum ersten Mal durch Peven und Persoz (1833) aus Malz extrahiert. Dann erkannten Schwarzer und O'Sullivan zwei Wirkungen im Malz, von denen die eine die Stärke verflüssigt und die andere sie verzuckert. Cuisinier ist der erste, welcher diese zwei Wirkungen zwei Enzymen, "Dextrinase und Maltase", zuschrieb. Nach ihm wirkt die Dextrinase direkt auf Stärke ein und spaltet sie zum Dextrin, und das letztere wird weiter erst durch die Maltase (nicht Maltase in der heutigen Auffassung) zur Maltose gespalten; aber die Maltase soll direkt auf Stärke selbst unwirksam sein. Durch die Arbeiten von Wijsman (1889), Duclaux, Beyerinck (1895) wurden diese zwei Enzyme durch ihre Dialysierbarkeit und durch ihre Empfindlichkeit gegen Wärme unterschieden. Später wurde diese Zwei-

Enzymtheorie von Fernbach (1910) durch seinen Versuch mit Malz bestätigt. Andererseits konnten Fränkel und Hamburg (1906) sowie Pribram (1912) durch die fraktionierte Dialyse zwei Enzyme, "das verflüssigende und das verzuckernde", trennen.

Es war andererseits durch Maquenne und Roux (1906) erwiesen, dass die Stärkekörner aus 15–20 % Amylopektin (Hülle) und 85–80 % Amylose (Inhalt) gebildet wurde. Diese beiden Substanzen unterscheiden sich durch ihre physikalischen und chemischen Eigenschaften. Wenn man die von diesen Autoren aufgestellte Kurve der durch Malzamylase gespaltenen Stärkekleister und Amylose betrachtet, kann man zweifellos zwei Perioden der Amylasewirkung im Falle des Stärkekleisters unterscheiden. Während die erste Periode, welche derselben der Verdauung der Amylose entspricht, schneller verläuft und schon nach höchstens einigen Stunden nahezu beendet ist, wobei vier Fünftel der gebrauchten Stärkemenge gespalten werden, dauert die zweite Periode mehrere Tage lange fort. Gerade aus dieser Tatsache glaubte man früher schliessen zu können, dass der Verzuckerungsprozess der Amylase dann vollendet sei, wenn die Verdauungsgeschwindigkeit vermindert ist. Bei dem betreffenden Versuche wurde aber überhaupt kein Amylopektin gebraucht; man vermutete den Verdauungszustand des Amylopektins nur indirekt aus der Vergleichung der Versuche mit Stärkekleister und Amylose. Dies genügt zweifellos nicht, um den Zustand der Verdauung des Amylopektins genau zu erkennen. Man wird dazu besser Amylopektin selbst anwenden.

Der Zwei-Enzymtheorie mangelt es nicht an Gegnern, welche die Ein-Enzymtheorie vertreten, wie Slosse und Limbosch (1908), Wohl und Glimm (1910). Wohl und Glimm sagen zusammenfassend über die durch Versuche teils bestätigten, teils neu gewonnenen Erfahrungen:

"Die Amylase stellt einen kolloidalen Katalysator von

einem den Eiweissstoffen ähnlichen chemischen Charakter dar, und wirkt stufenweise als ein Kolloid auf die Stärke durch die Adsorption; daher bestehen keine zwingenden Gründe, eine Mehrheit von Amylasen anzunehmen. Aber für die Wirksamkeit eines Enzyms müssen zwei Bedingungen zusammentreffen: die molekulare Bindung des Substrates und die Möglichkeit der reaktiven Einwirkung auf dasselbe. Und die Abhängigkeit der Reaktionsstufe von den äusseren Umständen braucht nicht auf chemischen Verchiedenheiten des Enzymes zu beruhen. Es kommen da als Ursachen ebensogut die verschiedenen Bedingungen für die Adsorption von Stärke und Dextrin an die kolloidale Amylase und Verschiedenheiten des Dispersionsgrades dieses Kolloides in Frage. In der Tat wirkt die Maltose hemmend auf die Amylase, aber die Hemmung der weiteren Verzuckerung durch die gebildete Maltose ist keine absolute, sondern eine Verzögerungserscheinung, entsprechend der Verminderung an freier Diastaseoberfläche. Die vorhandene Menge Amylase kann um so weniger an Dextrin gebunden sein und somit es weiterspalten, je mehr sie Maltose bindet. Diese Hemmungserscheinungen fallen aber bei grossem Diastaseüberschuss fort, weil dann trotz der Adsorption der Maltose noch genügend wirksame Diastaseoberfläche übrig bleibt; dasselbe ist natürlich der Fall bei Zugabe frischer Diastase. Ferner ist ohne weiteres gegeben, dass die Verzögerung durch Störung des Adsorptionsgleichgewichtes, also durch Fortschaffen oder Verdünnen der Maltose aufgehoben wird. Ausserdem greift durch Maltoseanhäufung festgelegte Diastase auch ohne Fortschaffung oder Verdünnung der Maltose eine neu zugesetzte Stärkemenge mit fast unverminderter Geschwindigkeit an (Brown und Morris). Die neu zugefügte heterogene Phase (verkleisterte Stärke) muss dann natürlich in der Lösung, aus der die Stärke verschwunden war, eine neue Enzymverteilung bedingen.'' Ausserdem erklärte auch Fouard die Amylasewirkung mit der Verschiedenheit des Dispersionsgrades.

Die Frage über eine Mehrheit von Amylasen könnte nur durch die Versuche mit einheitlichen Substraten wie reine Amylose und Amylopektin gelöst werden, weil ein und dasselbe Ferment eine ganz verschiedene Wirkungsweise gegen die verschiedenen Substanzen äussern könnte.

Es fällt mir schwer anzunehmen, dass die Präparate, welche bisher von vielen Forschern bei ihren Versuchen gebraucht wurden, ganz rein waren. Jedenfalls habe ich bisher nichts von Fxperimenten mit reinem Amylopektin gehört. Obwohl Gatin-Gruzewska (1908, 1912) es rein dargestellt hat, blieb ihre Beschreibung nur auf die Darstellungsmethode und seine Eigenschaften beschränkt, ohne auf den Versuch betreffs seiner Verdauung einzugehen. Zwar bezeichnet Maquenne (1908) ihr Präparat als ziemlich reines Amylopektin, es ist aber immerhin zweifelhaft, ob es wirklich ganz frei von Amylose war. Euler und Svanberg (1921) berichteten auch nur darüber, dass der Chemismus des Amylopektins erst dann festgestellt werden könne, wenn es in reinem Zustand dargestellt sei.

Nun habe ich seit zwei Jahren das Amylopektin nach der Methode von Gruzewska, welche von mir etwas modifiziert wurde, rein dargestellt und es zu weiterem Versuche gebraucht. Ausserdem habe ich Amylose nach meiner eigenen Methode rein hergestellt. Zuletzt kam die Darstellung von Amylase aus Grünmalz, auf welche ich besondere Sorgfalt verwandte. Alle Amylasen im Handel enthalten nach meiner Untersuchung auch Maltase und bilden aus Stärke nicht nur die Maltose sondern auch die Glukose.

Ich berichte in Nachfolgendem über die Darstellung von Amylose, Amylopektin und Amylase, und werde in nächster Mitteilung über das Resultat der damit angestellten Versuche berichten.

II. Die Darstellung der Stärke aus Kartoffeln.

Die gut abgewaschenen Kartoffeln werden fein zerrieben.

Nachdem man den erhaltenen Brei in einem Leinensack unter Wasser zwischen den Händen tüchtig ausgerieben hat, wird die ausgepresste Stärke mit einem grossen Quantum Wasser umgerührt und einige Stunden lang stehengelassen. Die schmutzige, rotbräunliche Flüssigkeit wird jetzt dekantiert und von den am Boden abgesetzten Stärketeilen kratzt man die dünne und teigige graue Oberschicht ab. Dann rührt man den Rückstand mit einer grossen Menge Wasser eine Stunde lang gut um und lässt ihn wieder stehen. Nachdem man dieselbe Manipulation 8–10 mal wiederholt und ganz weisse gute Stärke erhalten hat, behandelt man diese zum Zwecke der Entwässerung mit 90%igem Alkohol und nutscht sie ab, um sie zuerst in der Luft und dann im Exsiccator über Schwefelsäure zu trocknen.

Das Präparat stellt sich als weisses feines Pulver dar und 2.0 g davon gibt keine Spur von Stickstoff nach Kjeldahlscher Methode.

III. Die Darstellung der Amylose.

1) Die passende Temperatur und Zeitdauer bei Kleisterbereitung aus Kartoffelstärke zur Trennung der Amylose vom Amylopektin.

Um auf ideale Weise die Amylose ganz amylopektinfrei erhalten zu können, ist es dringend notwendig, dass bei der Kleisterbereitung die Hülle von Amylopektin möglichst nicht zerkleinert wird, sondern dass sie möglichst ihre sackähnliche Form behält, indem der Inhalt nur aus einer Stelle der Hülle nach aussen heraus gelöst wird. Von der technischen Seite betrachtet ist es ebenso wichtig, dass die Amyloselösung möglichst konzentriert und in grosser Menge erhalten wird.

Um die besten Bedingungen zu diesem Zwecke zu erproben, habe ich zuerst die Formveränderungen des Sackes bei verschiedener Temperatur und Kochdauer bei der Kleisterbereitung unter dem Mikroskop mit einem Tropfen von 1/5

Lugolscher Lösung verfolgt. Daneben wurde die Menge und Konzentration der nach 24 stündigem Stehen erhaltenen Amyloselösung bestimmt. Diese Bestimmung wurde nach der folgenden einfachen Methode ausgeführt: vom 1% igen Kleister, welcher durch einstündiges Kochen bei 75°C. bereitet wurde, wird nach 24 stündigem Stehen die obenschwimmende Flüssigkeit sorgfältig abfiltriert; 5 ccm hiervon, mit einem Tropfen von 1/5 Lugollösung vermischt, werden als Originallösung betrachtet, wonach andere Lösungen kolorimetrisch bestimmt werden. Zu gleicher Zeit werden 200.0 ccm des Filtrats 2 Stunden lang mit 10 ccm konzentrierter Salzsäure im Wasserbade und mittels des Rückflusskühlers hydrolysiert und dann mit NaOH neutralisiert. Nachdem man den Zuckergehalt der Flüssigkeit nach Momosescher Modifikation der Pavy-Kumagawa-Sutoschen Zuckerbestimmungsmethode (Momose, 1906) bestimmt hat, rechnet man ihn auf den Stärkegehalt durch Multiplikation mit 0.9 um.

Was den gewonnenen Stärkekleister betrifft, so wird bei höherer Temperatur und durch längeres Kochen das Amylopektin-säckchen natürlich mehr zerkleinert und die Amylose mehr konzentriert als bei niedrigerer Temperatur und kürzerer Kochdauer. Folglich braucht der auf jene erste Weise bereitete Kleister auch längere Zeit, bevor er sich niederschlägt; ja, das Niederschlagen erfolgt dabei überhaupt nur unvollständig. Hierdurch wurde ich schliesslich veranlasst, die Niederschlagsgeschwindigkeit unter verschiedenen Bedingungen genauer zu untersuchen. Zu dem letzten Zwecke wurden je 20 ccm von 1% igem, bei verschiedenen Temperaturen bereiteten Kleister in graduierte, gleichkalibrierte Probiergläser von etwa 25 ccm gefüllt und 24 Stunden lang stehengelassen. Das Produkt der Volumzahl (a) der oberen klaren Lösung mit der Konzentrationszahl der Amylose (b), welche "effiziente Menge" genannt wurde, wurde in folgender Tabelle angegeben.

TABELLE I.

Die effiziente Menge der Amylose bei verschiedener Temperatur und Zeitdauer der Kleisterbereitung.

Temperatur	Zeitdauer	(a) Volumzahl	(b) Konzentr.	Effiziente Menge
	Stunde	*ccm*	*g/dl*	
80°	1	6.3	0.084	0.53
	2	3.0	0.108	0.32
75°	1	11.0	0.060	0.66
	2	7.5	0.084	0.63
70°	1	15.0	0.048	0.72
	2	13.0	0.072	0.94
	3	13.0	0.096	1.25
65°	1	15.7	0.036	0.57
	2	15.3	0.048	0.73
	3	15.0	0.048	0.72
	4	15.0	0.048	0.72

Aus dem Ganzen geht hervor, dass die "effiziente Menge" der Amylose bei 3 stündiger Kochdauer und 70°C. am grössten ist; dieser Koeffizient ist aber für meinen Zweck nicht so geeignet, weil die Formveränderung des Säckchens dabei etwas zu gross ist. Ich bereitete daher den Stärkekleister von nun an durch 2 stündiges Erwärmen bei 70°C.

2) Die Darstellung der Amylose.

25g Kartoffelstärke werden in 100 ccm Wasser suspendiert und unter stetem Umrühren in 2.4 *l* Wasser von 70°C. hineingegossen. Das Gemenge wird dann bei 70°C. unter Umrühren alle 10 Minuten 2 Stunden lang erwärmt. Dann wird der Kleister 24 Stunden lang stehengelassen und die obenschwimmende Lösung mit glattem Filterpapier klar filtriert. Das Filtrat wird im Wasserbade unter stark vermindertem Druck auf 1/8 bis 1/10 Volum eingedickt.

Man filtriert diese dickflüssige Lösung mit Faltenfilter möglichst schnell und fügt ihr danach sofort 90% igen Alkohol solange zu, bis die Endkonzentration des letzteren ca 50% beträgt. Diese Manipulation muss möglichst schnell ausgeführt werden, da sich einmal herausstellte, dass die eingedickte Lösung ohne Zusatz von Alkohol nach dem Erkalten leicht zu einer klumpigen Masse wird. ("Retrogradation" nach Maquenne und Roux).

Man nutscht den Niederschlag nach einigen Tagen ab, behandelt ihn nach und nach mit immer stärkerem Alkohol, und schliesslich mit absolutem Alkohol und Äther. Nach dem Eintrocknen wird er pulverisiert.

Es ergibt sich ein ganz weisses Pulver, welches sich im Verhältnis von etwa 0.5% in heissem Wasser löste. Die heisse Lösung muss man bis zur Erkaltung beständig umrühren und dann erst filtrieren, da sie sonst leicht Klümpchen bildet.

IV. Die Darstellung des Amylopektins.

Im grossen und ganzen behandelte ich die Stärke nach der zweiten Darstellungsmethode von Frau Gatin-Gruzewska (1912), indem ich an einigen Punkten noch etwas zu verbessern strebte. Ich habe dabei sowohl die Einwirkungsdauer von 1% iger Natronlauge auf die Stärke als auch die Stärke der Umrührung des Kleisters reguliert, damit die sackförmige Hülle der Stärkekörner möglichst gut erhalten bleiben konnte. Durch das mehrmalige Auswaschen mit Wasser des so behandeltens Säckchens konnte ich Amylopektin erhalten, welches mikroskopisch fast ganz frei von Amylose befunden wurde. Die Beschreibung der Methode ist folgendermassen.

Man giesst 100 ccm von 20% iger Stärkesuspension unter Umrühren in 900 ccm Wasser, welches 10 g Natronlauge enthält; man rührt dabei zunächst 2 Minuten lang ziemlich schnell und darauf 3 Minuten lang langsamer um. Die

Flüssigkeit wird schon nach 1 Minute halbdurchsichtig, sogar dickflüssig. Am Ende der 5 Minuten fügt man 3 Liter Wasser zu und rührt sie weiter 10 Minuten lang mittelst des mit Wasserturbine verbundenen Rührwerks (60–100 Umdrehungen pro Minute) um. Dabei muss man allzu schnelles Umrühren vermeiden, weil der Sack sonst leicht zerkleinert werden könnte. Zuletzt wird die Flüssigkeit unter Zusatz von Essigsäure (36%) schwach sauer gemacht; dabei wird sie getrübt, indem das Amylopektin zusammenschrumpft. Nach 10 Minuten langem Rühren setzt man eine grosse Menge Wasser hinzu und rührt wieder 30 Minuten lang um; dann lässt man die Flüssigkeit nach Zusatz von Chloroform stehen. Nach 24 Stunden wird sie dekantiert, der Niederschlag von neuem mit einer grossen Menge Wasser umgerührt und wieder stehengelassen. Dies muss man bis zu dem Zeitpunkte (etwa 8 mal) wiederholen, wo das Amylopektin ganz von Amylose befreit worden ist, was man von folgenden zwei Punkten erkennen kann: nämlich dadurch, dass erstens der Niederschlag durch Zusatz von 1/5 Lugollösung rein rotviolett gefärbt wird und zweitens unter dem Mikroskop sich keine blaue Verfärbung innerhalb und ausserhalb des Sackes beobachten lässt. Dann fügt man dem Niederschlag das 3 bis 4 fache Volumen Alkohol (90%) hinzu, behandelt ihn nach und nach mit Alkohol von steigender Konzentration, zuletzt mit absolutem Alkohol und Äther. Nach dem Eintrocknen wird er im Paraffin-exsiccator aufbewahrt, um auch die letzten Spuren von zurückbleibendem Äther zu entfernen.

Es ist ein weisses Pulver und enthält 0.074% Phosphor (oder 0.24% Phosphorsäure) nach Bloorscher Methode (1918).

V. Die Bereitung des Gerstenmalzes.

Zur Malzbereitung wurde Imperialgerste ("Golden melon") gebraucht. welche mittelst des Auswahlwerks in gleicher

Grösse (2.5×3 mm) ausgewählt wurde. Ich erprobte die Keimfähigkeit der Gerste einfach durch Legen zwischen staubfreie, feuchte Filterpapierschicht (Leyser, i) und stellte fest, dass 95 Prozent der Körner keimten.

Es ist ein anerkannter Grundsatz, dass man zur Malzbereitung die Gerste zuerst gut auswaschen muss. Dann folgt das Weichen.

Das Weichen. Als Reinigungsmittel der Gerste vor dem Weichen wurde die gesättigte Kalklösung, welche durch die Mischung von 1 kg Kalk zu 760 kg Wasser hergestellt wurde, verwendet (Leyser, ii).

Nachdem man die ausgewaschene Gerste 9 Stunden lang in Kalklösung eingetaucht gelassen hat, tauscht man diese mit Wasser um, und ersetzt das letztere zuerst nach 12 Stunden, dann alle 6 Stunden. Im ganzen dauert das Weichen 60 Stunden lang bei etwa 16°C. Bei niedrigerer Temperatur wird die Weichdauer aber bis auf 72 Stunden verlängert (Vergl. Thausing, 1907). Schliesslich ist das Weichen vollendet (Leyser, iii): wenn die Hülse sich leicht von dem mehligen Korne ablöst und sich da zu öffnen scheint, wo der Wurzelkeim hervorbrechen soll; wenn die Hülse aufspringt, indem man das Korn an den Spitzen zwischen Daumen und Zeigefinger—ohne den Fingern wehe zu tun—zusammendrückt und der Mehlkörper sich dabei elastisch erweist; wenn sich das Korn über den Nagel biegen lässt; wenn das Korn beim Durchbeissen nur geringen Widerstand leistet; wenn das Korn beim Durchschneiden der Quere nach mit einem scharfen Messer, und zwar in der Mitte, bis auf einen kleinen weissen Kern im Zentrum eine gelbliche Schnittfläche zeigt.

Die Germination. Man bringt die geweichte Gerste in den nach Saladins Keimapparat von mir ausgedachten Keimtopf (Fig. 1 und 2, K) hinein, in welchen feuchte Luft mittelst Wasserstrahlpumpe hineingesaugt wird. Zur Keimung der Gerste sind 3 Bedingungen unbedingt notwendig, nämlich

Sauerstoff, Wärme und Feuchtigkeit; dabei kann Kohlensäure schon so nachteilig einwirken, dass die Keimfähigkeit um ein Drittel vermindert wird, wenn die Luft 4% Kohlensäure enthalten hat (Chabrié, 1908).

Die Richtung des Luftstromes innerhalb des Topfes wird mittels des von mir entworfenen, besonderen Glashahnes (H) 2-3 mal täglich gewechselt, zur selben Zeit auch die Gerste darin behutsam umgerührt. Nach 6 Tagen bei etwa 16°C., wenn der Blattkeim etwa 3/4 der Länge des Korns im Innern und die Wurzelkeime die $1\frac{1}{2}$ fache Kornlänge erreicht haben, wird der Keimprozess unterbrochen, indem man trockne Luft 12 Stunden lang zugeführt. Auf diese Weise habe ich das Grünmalz erhalten und es zur Darstellung der Amylase ohne Darren gebraucht.

VI. Die Darstellung der Amylase.

Bei der Darstellung der Amylase habe ich eine in der Hauptsache auf Lintner (1898) und Wroblewski beruhende und von mir etwas modifizierte Methode befolgt.

a) Zuerst benutzte ich die Methode von Lintner. Ein Quantum des oben erhaltenen Grünmalzes wird fein zerrieben und mit der vierfachen Menge von 20 % igem Alkohol versetzt. Um das Gemenge gut mazerieren zu lassen, wurde es mittels elektrisch betriebenem Rührwerke 2 Stunden lang schnell umgerührt. Man lässt die Masse dann im ganzen 24 Stunden lang stehen und ausziehen, trennt die Flüssigkeit vom Malze mittels eines Leinensackes, filtriert sie mit doppeltem Faltenfilter und setzt alsdann dem Filtrat die doppelte Menge absoluten Alkohols (oder das $2\frac{1}{2}$-fache Volumen 90 %igen Alkohols) zu Es bildet sich hierbei ein flockiger Niederschlag; man trennt die klare Flüssigkeit mit dem Siphon, bringt den Niederschlag auf das doppelte Faltenfilter und wäscht ihn mit Alkohol und Äther einige Male aus.

b) Dann folgte die Methode von Wroblewski, welche

darauf beruht, die enthaltene Pentose (nämlich Araban) durch fraktionierte Fällung mit dem Salze zu entfernen.

Der oben in a erhaltene Niederschlag wird mittels Spatel in einen Mörser getan, bevor er noch getrocknet ist, mit destilliertem Wasser zerrieben, bis er vollständig aufgelöst ist, dann mit Faltenfilter filtriert.

Man fügt diesem Filtrat nach und nach so viel schwefelsaures Ammoniak in Substanz bei, bis es 50 % davon enthält; dabei beginnt sich die Lösung zu trüben. In Wirklichkeit macht es keinen Unterschied, ob man dieses Salz in der Substanz oder als Lösung gebraucht, weil in beiden Fällen der entstandene Niederschlag reversibel ist. Nach einiger Zeit setzt sich ein Niederschlag, bestehend aus kleinen gelblichen Flocken, ab; man bringt ihn auf doppeltes Faltenfilter und wäscht ihn mit einer Lösung von 54 %igem schwefelsauren Ammoniak aus.

c) Zuletzt kam die von mir aufgestellte weitere Behandlung in Anwendung. Weil der hier erhaltene Niederschlag noch eine zu grosse Menge Salz enthält, ist es nötig, diese wässerige Lösung einmal zu dialysieren, um das Salz und sogar jeglichen Rest von Zucker zu entfernen.

Der in b) erhaltene Niederschlag wird in einer kleinen Menge destillierten Wassers gelöst, mit glattem Filterpapier filtriert und dann mit dem Kollodiumdialysator 2 Stunden lang in fliessendem Wasser dialysiert. Die Dialyse darf nicht zu lange Zeit dauern, weil dabei auch die Amylase verloren gehen kann; ich habe selbst einmal wahrgenommen, dass der Inhalt des Sackes schon nach 2 tägiger Dialyse ganz unwirksam war. Nach beendeter Dialyse filtriert man den Niederschlag mit Faltenfilter, setzt dem Filtrat das 4 bis 5-fache Volumen Alkohol (90 %) zu und nutscht den dadurch entstandenen Niederschlag nach einiger Zeit ab und behandelt ihn mit absolutem Alkohol und Äther. Nach dem Trocknen wird er pulverisiert und im Paraffin-exsiccator, vor Sonnenlicht geschützt, aufbewahrt.

Aus einem Quantum Grünmalz, welches aus 3 Sho (etwa 6 *l*) Gerste hergestellt wurde, konnte ich durch einmaliges Verfahren nur höchstens etwa 4 g Amylase bekommen; aber durch mehrmaliges Wiederholen desselben Verfahrens erhielt ich eine gesamte Ausbeute von 43 g.

Das Präparat ist ein braun-gelbliches Pulver; es löst sich ziemlich schwer in kaltem Wasser, daher muss es mittelst des mit elektrischem Motor verbundenen Rührwerks sehr schnell 1 Stunde lang umgerührt werden, bis es eine brauchbare Lösung gibt. Zum Versuche filtrierte ich sie einmal vor der Anwendung; ihre Wirksamkeit von 1 %iger Lösung ist nach Robertscher Methode D = 0.7. Diese Lösung enthält aber 0.0249 g/dl von reduzierbarer Substanz.

VII Die Bestimmung der Maltose.

Zur Bestimmung der bei der Spaltung der Stärke gebildeten Maltose ist die Momosesche Methode ganz geeignet. Da die Reduktionsfähigkeit der Maltose gegen Kupferlösung bei weitem kleiner als diejenige der Glukose ist und zur vollständigen Entfaltung ihrer Reduktionskraft längere Zeit braucht, ist es unbedingt nötig, geeignete Bedingungen aufzufinden.

Ich stellte daher durch die Versuche eine geeignete Kochdauer für Maltose (Merck) als 5 Minuten fest. Wenn man nach dem Sieden noch ganz schwach 2 Minuten lang weiter kocht, lässt sich der Kolben ganz von Luft befreien. Ich brauchte immer zur Bestimmung auf 20.0 ccm Kupferlösung etwa 25 ccm Alkalilösung statt 20 ccm bei originaler Menge, weil der Kolbeninhalt weit längere Zeit gekocht werden musste.

Die von mir gebrauchte Maltose (Merck) ist das pulverisierte Krystall; weil sie aber nicht ganz eingetrocknet worden war, ist die durch Bestimmung erkennbare Konzentration weit kleiner als das zur Bereitung der Lösung gebrauchte Quantum. Da die Maltose gegen Wärme sehr empfindlich ist und schon bei 100° C. eine innere Veränderung erleidet, ohne in ihrem Aussehen irgendeine Besonderheit zu zeigen, habe ich sie nicht

besonders entwässert, sondern unverändert für meine Versuche gebraucht und seine Menge als Glukose nach Hydrolyse nach Allihnscher Methode bestimmt.

(1) Die Bestimmung der Maltose nach Momoseschen Methode.

Zuerst stellt man 0.5 %ige Maltoselösung her und kocht sie nach Eintropfen von Lösung mit der Kupferlösung verschieden lange Zeit, um die entsprechende Kochdauer erkennen zu können. Das Resultat stellte sich wie folgt:

Kochdauer *Min.*	Maltose gefunden *g/dl*
1	0.1813
2	0.1875
3	0.1896
4	0.1917
5	0.1917

Daher sind 4 Minuten Kochdauer für Maltose genügend, aber man soll lieber 5 Minuten kochen.

(2) Die Vergleichung der Reduktionsfähigkeit der Maltose und Glukose.

Zuerst stellt man 0.5 %ige Maltoselösung her; auf je 100.0 ccm derselben wurde einerseits 2.5 ccm, andererseits 5 ccm konzentrierter Salzsäure hinzugefügt, und die Lösung 1-2 Stunden lang mit dem Rückflusskühler im kochenden Wasserbade hydrolysiert; dann wurde der Zuckergehalt nach Neutralisierung mit NaOH methodenweise bestimmt (a, b und c).

a) 100.0 ccm Lösung + 2.5 ccm konz. HCl

Dieses Gemisch wurde 2 Stunden lang hydrolysiert.

Die Menge der reduzierbaren Substanz ...0.4136 g/dl

b) 100.0 ccm Lösung + 5 ccm konz. HCl
2 Stunden lang hydrolysiert.
Die Menge der reduzierbaren Substanz....0.4136 g/dl

c) 100.0 ccm Lösung + 2.5 ccm konz. HCl
1 Stunde lang hodrolysiert.
Die Menge der reduzierbaren Substanz....0.3822 g/dl

Daher ist es bei 100.0 ccm 5 %iger Maltoselösung unbedingt notwendig, mit 2.5 ccm konzentrierter Salzsäure 2 Stunden lang im kochenden Wasserbade zu hydrolysieren.

d) Andererseits wurde die Reduktionsfähigkeit der Mal-toselösung selbst bestimmt.
Die Menge der reduzierbaren Substanz....0.2111 g/dl

Daraus ist das Verhältnis der Reduktionsfähigkeiten von Maltose zu Glukose bei Momosescher Bestimmungsmethode 51.04%.

VIII Die Hydrolyse der Amylose und des Amylopektins.

Im vorigen Paragraph habe ich geschildert, dass es zur Hydrolyse der 0.5 %igen Maltoselösung unumgänglich nötig ist, 100 ccm derselben Lösung mit 2.5 ccm konzentrierter Salzsäure 2 Stunden lang zu kochen. Bei Amylose und Amylopektin liegt die Sache etwas anders, weil die beiden Substanzen komplizierter als Maltose sind.

Ich habe dann nach Momosescher Methode die durch Hydrolyse erhaltene Flüssigkeit mit Kupferlösung auf zweierlei Weise, 1 Minute und 5 Minuten lang, gekocht. Dabei müssen beide Resultate ungleich sein, wenn die Hydrolyse noch nicht ganz vollendet und noch irgendeine Menge von Maltose unverändert geblieben ist, weil die Reduktionskraft der Maltose schwächer als diejenige der Glukose ist. Daraus kann man schliessen, dass die Hydrolyse vollständig ist, wenn die Resultate in beiden Fällen gleich sind.

Die Hydrolyse der Amylose. Man löst 2.5 g Amylose in 500 ccm kochenden Wassers und filtriert die Lösung nach Abkühlung; sie enthält keine reduzierbare Substanz.

a) 100.0 ccm Lösung + 2.5 ccm konz. HCl
Man hydrolysiert sie im Wasserbade 2 Stunden lang.

Kochdauer Min.	reduz. Substanz g/dl
1	0.3037
5	0.3455

b) 100.0 ccm Lösung + 5 ccm konz. HCl
1 Stunde lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.3455
5	0.3822

c) 100.0 ccm Lösung + 5 ccm konz. HCl
2 Stunden lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.4293
5	0.4293

(d 100.0 ccm Lösung + 5 ccm konz. HCl
3 Stunden lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.4293
5	0.4293

Daher ist es zur Hydrolyse solcher Amyloselösung notwendig, mit 5 ccm konzentrierter Salzsäure wenigstens 2 Stunden lang zu kochen.

Die Hydrolyse des Amylopektins. Man löst 2.5 g Amylopektin in 500 ccm kochenden Wassers, filtriert die Lösung nach Abkühlung. Sie enthält keine reduzierbare Substanz.

a) 100.0 ccm Lösung + 2.5 ccm konz. HCl
2 Stunden lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.2880
5	0.3351

b) 100.0 ccm Lösung + 5 ccm konz. HCl
1 Sunde lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.3141
5	0.3613

c) 100.0 ccm Lösung + 5 ccm konz. HCl
2 Stunden lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.4031
5	0.4031

d) 100.0 ccm Lösung + 5 ccm konz. HCl
3 Stunden lang hydrolysiert.

Kochdauer Min.	reduz. Substanz g/dl
1	0.4031
5	0.4031

Daher ist das Resultat ganz dasselbe wie bei der Hydrolyse der Amylose.

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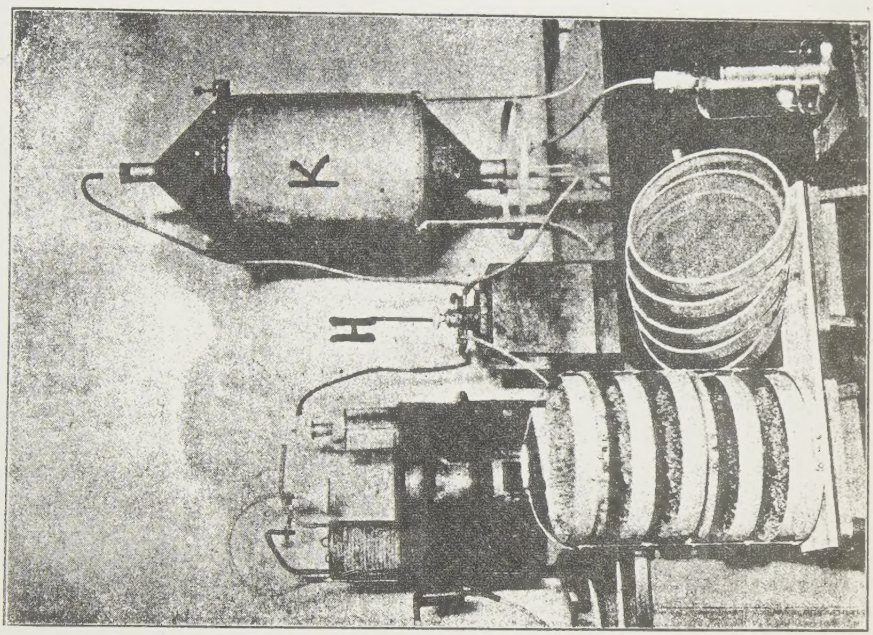

Fig. 2.

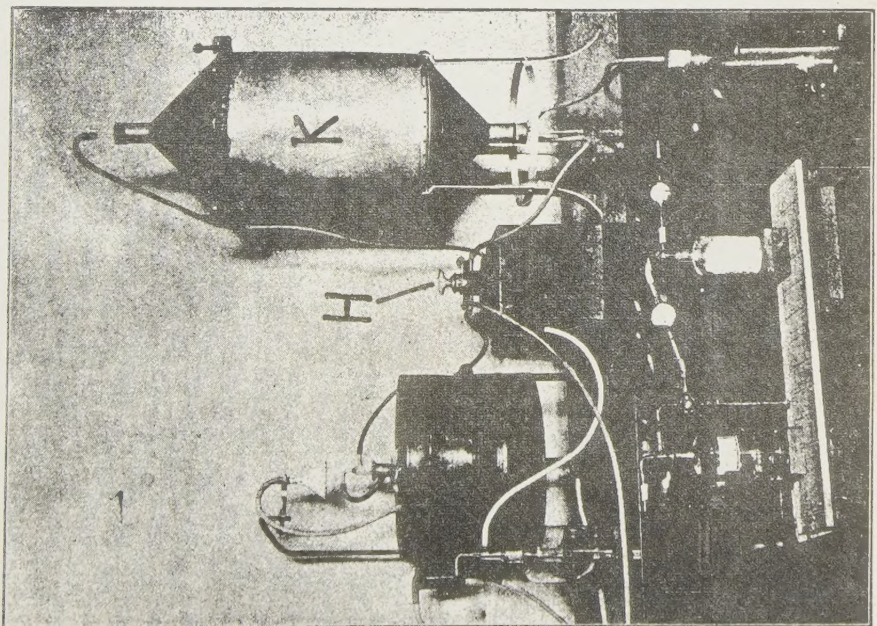

Fig. 1.

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